Pathogen control at the intestinal mucosa – H$_2$O$_2$ to the rescue

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**ABSTRACT**

Intestinal infections are a global challenge, connected to malnutrition and inadequate hygiene in developing countries, and to expanding antibiotic resistance in developed countries. In general, a healthy host is capable of fighting off gut pathogens or at least to recover from infections quickly. The underlying protective mechanism, termed colonization resistance, is provided by indigenous commensal communities (microbiota) that are shaped and aided by the host’s epithelial and innate immune system. Commensal-pathogen interactions are governed by competition for a suitable niche for replication and stable colonization, nutrient availability, species-specific alterations of the metabolic environment, changes in oxygen tension and release of chemicals and proteinaceous toxins (bacteriocins). This protective intestinal milieu is further reinforced by antimicrobial factors and chemicals secreted by the epithelial barrier, by dendritic cell sensing and by homeostasis between T-cell subsets (Treg/Th17) in the lamina propria. The 3 players (host-microbiota-pathogen) communicate via direct interactions or secreted factors. Our recent manuscript illustrates that reactive oxygen species (ROS) are an integral part of colonization resistance and should be considered an interkingdom antivirulence strategy.

**KEYWORDS**

antivirulence; C. rodentium; defensive symbiosis; lactobacilli; NADH flavin Reductase; LEE pathogenicity island; NADPH oxidase; reactive oxygen species (ROS)

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**Reactive oxygen species as chemical signals**

ROS are essential chemicals for resolving infectious disease and as we appreciate now, for modifying the microbiota composition and hence colonization resistance. The importance of the phagocyte NADPH oxidase (NOX2 complex) as defense mechanism in infections is apparent in chronic granulomatous disease (CGD), an inherited immunodeficiency caused by inactivating variants of CYBB (NOX2) and associated complex components. CGD patients present with life-threatening bacterial and fungal infections as superoxide produced by NOX2 is required for microbial killing by phagocytes. The role of NADPH oxidases expressed in the barrier epithelium of the intestine, namely NOX1, NOX4 and DUOX2, remains less defined. Studies in Drosophila, C. elegans and mice connected DUOX to microbial clearance, redox signaling and epithelial cell renewal, while NOX1-derived superoxide was required for redox signaling in intestinal epithelial cells (IECs), restitution and wound repair. DUOX2 expression is influenced by the presence of microbiota but the particular stimuli leading to upregulation and activation of mammalian DUOX2 have not yet been identified. Epithelial oxidases are recruited, upregulated, and activated when IECs sense intestinal pathogens, presumably by TLR/NLR signaling. The generated superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are used for intracellular redox signaling and as diffusible messenger (H$_2$O$_2$) in the extracellular space. The H$_2$O$_2$ concentration achievable in the large intestinal compartment is too low to act as a bactericide, but cell-based studies showed a pathogen repellent effect for H$_2$O$_2$ that reduced bacterial invasion. Released H$_2$O$_2$ at nano- to low micromolar concentrations diffuses into extracellular bacteria and alters gene transcription and signal transduction. Intestinal pathogens rely on tyrosine phosphorylation for polysaccharide biosynthesis and metabolic processes. H$_2$O$_2$-induced oxidative conversion of phosphotyrosine residues to protein-bound...
DOPA markedly reduced the phosphotyrosine content of bacterial enzymes and downregulated pathogen virulence. IEC-generated ROS have been linked to bacterial recognition responses, inflammasome activation, and autophagy. A general role for NOX enzymes in regulating macroautophagy in multiple cell types has been described while consensus is evolving that mitochondria-derived ROS mediate NLRP3 activation. These multiple ROS signaling streams, and in particular H$_2$O$_2$ as relatively stable messenger, pose questions about the hierarchy of responses in defending the host with these chemicals. This addendum provides a synopsis of our recent work delineating the consequences of ablated NOX activity for intestinal homeostasis, microbial communities and pathogen defense and insights into H$_2$O$_2$ generation by probiotic bacteria.

**The paradox of host welfare and NOX-ROS deficiency**

Understanding the ultimate outcome of the host-commensal-pathogen interaction when NADPH oxidase enzymes for host oxidant production are absent, is often complicated by compensation or redox signaling involving other oxidases and ROS sources. We addressed this challenge by deleting p22$^{phox}$ (Cyba$^{-/-}$), the obligatory dimerization partner of all NOX family isoforms expressed in mice (NOX1−4), in the intestinal epithelium (ΔIEC) or in the whole body. Comparison with NOX2 knockout mice (Cybb$^{-/-}$) permits pinpointing observed effects to the epithelial barrier, the innate immune system (expressing mainly NOX2), or both compartments including other tissues. ROS generation in the targeted compartments was minimal (IEC) or abolished (neutrophils), but overall crypt architecture of all mouse strains was comparable to wildtype mice. NOX-generated O$_2^*/$H$_2$O$_2$ has been linked to goblet cell autophagy, NLRP6 signaling and maintenance of the mucus layer, but goblet cell numbers, the number and thickness of the dense mucus layer were not altered in p22$^{phox}$- or NOX2-deficient mice. To study the host response of p22$^{phox}$-deficient mice in intestinal infection without disturbing the microbiota is only possible with selected pathogens, for example with the murine pathogen *Citrobacter rodentium*, which mimics human EPEC/EHEC infections.

Colonization resistance might be influenced by epithelial NOX/DUOX, by NOX2-induced signaling in dendritic cells, neutrophils, macrophages or T/B lymphocytes, or might not be dependent on NOX-derived ROS at all. Comparing mice with abolished NOX activity in different compartments should answer this question. If the absence of NOX-derived ROS leads to dysbiosis, the colonization resistance should be negatively affected and *C. rodentium* disease severity would increase dramatically in immunocompromised hosts (NOX2 or p22$^{phox}$ complete deficiency). Surprisingly our work showed that colonization resistance was substantially increased by both, p22$^{phox}$ deficiency in the epithelium and in the whole body, while NOX2 ablation was inconsequential and resembled disease progression in wildtype mice. Inactivation of epithelial NOX mimicked whole body NOX inactivation, both resulting in comprehensive protection of the host against *C. rodentium* or *Listeria monocytogenes*. On the other hand, when colonization resistance was abolished by multi-antibiotic treatment, superoxide produced by NOX2 was essential for host survival. These results confirm that immune cell-derived superoxide is critical for gut pathogen defense when the microbiota cannot provide colonization resistance. However, it is surprising that eliminating NOX function in the mucosal barrier confers an advantage for host health.

**Recruitment of an auxiliary ROS source**

Microbiota-host relationships are often mutualistic with both providing benefits to each other. In contrast to dysbiosis, the blooming of symbiotic bacteria which display defensive attributes (defensive symbiosis) could protect a vulnerable host. Closer examination of p22$^{phox}$-deficient mice revealed some changes in colonic and ileal mucus composition that may select for colonization with particular microbial species. Further, shedding of *C. rodentium* was enhanced in the initial stages of infection, indicating a less hospitable environment for the pathogen. The enhanced colonization resistance toward *C. rodentium* was transferrable to wildtype mice, indicating a shift in the microbiota composition. Microbiota analysis revealed a marked enrichment in lactobacilli in p22$^{phox}$-deficient, but not in NOX2 knockout mice.

Lactobacilli are considered beneficial probiotics, which colonize intestinal surfaces and provide health
benefits not only in infections, but also in inflammatory bowel diseases.\textsuperscript{20-22} The prevalence of \textit{L. johnsonii} in the colitogenic phenotype of IL-10 knockout mice\textsuperscript{23} or the proinflammatory phenotype of the \textit{L. rhamnosus} GG cell wall polymer lipoteichoic acid\textsuperscript{24} suggests distinct species differences in regard to their preferred environment, possibly even promoting inflammation. Association of IL-10-deficient mice with \textit{L. reuteri} or \textit{L. plantarum} suppressed colitis,\textsuperscript{25} supporting the notion of biologically relevant differences between \textit{Lactobacillus} strains. Mice with \textit{p22}\textsubscript{phox} deficiency harbored mainly \textit{L. reuteri} and \textit{L. murinus},\textsuperscript{16} both considered beneficial. Mono-association of microbiota depleted NOX2 knockout mice with \textit{L. reuteri} or \textit{L. murinus} prevented death from systemic, lethal \textit{C. rodentium} infection.\textsuperscript{16} This protection is likely due to improved mucosal barrier integrity in conjunction with changes in the T lymphocyte compartment.\textsuperscript{26,27} Probiotic bacteria can stabilize IEC junctional complexes by inducing increased expression of occludin and cingulin genes, redistribution of the tight junction protein ZO-2 and downregulation of the leaky protein claudin-2.\textsuperscript{28} While dissemination of \textit{C. rodentium} to other organs was curtailed in NOX2-deficient mice, \textit{Lactobacillus} mono-association did not provide complete colonization resistance.\textsuperscript{16} However, continuous oral treatment with a single \textit{Lactobacillus} strain provided colonization resistance to \textit{C. rodentium}-infected wildtype mice harboring their indigenous microbiota, similar to the protection observed after transplantation of microbiota collected from \textit{p22}\textsubscript{phox}-deficient mice.\textsuperscript{16}

How can lactobacilli achieve such effective protection of the host? The beneficial effects of lactobacilli have been linked to cell wall components and secreted factors such as bacteriocins, lactic acid, chemicals (e.g. reuterin) and H$_2$O$_2$. Our studies indicated that protection was not dependent on one particular \textit{Lactobacillus} species, and that colonization resistance was critically dependent on the host epithelium. Both, lactobacilli and the host have the chemical H$_2$O$_2$ as common defensive strategy. H$_2$O$_2$ will be released by NOX/DUOX oxidases upon pathogen contact only transiently, while lactobacilli can generate H$_2$O$_2$ for as long as sufficient oxygen is available. A 70\textmu m zone of relative oxygenation adjacent to the intestinal mucosa, caused by diffusion from the capillary network at the tips of villi, provides oxygen to lactobacilli colonizing the loose mucus layer.\textsuperscript{29} The achievable H$_2$O$_2$ production by the mucosa versus lactobacilli cannot be measured quantitatively \textit{in vivo}, but is comparable \textit{in vitro}. Thus, if H$_2$O$_2$ is required for protection of the host, and in particular the ROS-deficient host, then lactobacilli will be more efficient than the barrier epithelium. In accord, only a \textit{Lactobacillus} strain with intact H$_2$O$_2$ production was capable of providing host protection in \textit{C. rodentium} infection.\textsuperscript{16,30}

**H$_2$O$_2$ generation by lactic acid bacteria**

Hydrogen peroxide production is a common characteristic in the lactobacillales clade that comprises the lactic acid bacteria (LAB). Species belonging to the \textit{L. acidophilus} group such as the yoghurt isolate \textit{L. delbrueckii} subsp. bulgaricus,\textsuperscript{31} the vaginal lactobacilli \textit{crispatus}, \textit{gasseri} and \textit{jensenii},\textsuperscript{32} and the gut isolate \textit{L. johnsonii}\textsuperscript{30} are known to generate copious amounts of H$_2$O$_2$ under aerobic conditions. Other known H$_2$O$_2$ producers are found among the pathogenic streptococci, such as \textit{Streptococcus pneumoniae} (via SpxB) and \textit{S. pyogenes} (via LacD). The 2 \textit{Lactobacillus} strains isolated from the intestine of \textit{p22}\textsubscript{phox}-deficient mice, \textit{L. reuteri} and \textit{L. murinus}, are also capable of producing H$_2$O$_2$ and addition of catalase to cell-free extracts abolished their antagonistic effect toward \textit{C. rodentium}.\textsuperscript{16}

H$_2$O$_2$ is far from harmless for the bacteria producing it. Under atmospheric conditions the species belonging to the \textit{L. acidophilus} group can accumulate up to 1 mM of H$_2$O$_2$ in their surroundings, which leads to oxidative stress, premature growth stagnation and viability loss. In several streptococci, the peroxide forming capacity was associated with the lactate oxidation pathway, which is induced after glucose depletion.\textsuperscript{33,34} In this pathway lactate is converted to acetate through the sequential activity of lactate oxidase or NAD-dependent lactate dehydrogenase, pyruvate oxidase and acetate kinase (Fig. 1). Although this yields one additional ATP moiety, acetate production through this pathway is generally associated with dramatic viability loss during aerobic stationary phase.\textsuperscript{35-37} While lactate production from glucose is a redox neutral process, acetate production from pyruvate requires the additional oxidation of NADH. In the absence of a functional respiratory chain (LAB are hemin auxotrophs) this oxidation occurs through a H$_2$O or H$_2$O$_2$ forming NADH oxidase. In species such as \textit{L. delbrueckii}, \textit{L. johnsonii} and \textit{L. panis}, NADH oxidation was found to
be the main source of ROS production. In *L. lactis* a H₂O forming NADH oxidase released superoxide radicals, which spontaneously dismutated to H₂O₂. Many other cellular components, such as quinones and proteins with solvent-exposed flavin moieties can auto-oxidise leading to superoxide formation. An example is the univalent auto-oxidation of dimethylmenaquinones in *Enterococcus faecalis*, which is the main source of extracellular superoxide released by this species.

An important aspect of a biologic makeup that allows for H₂O₂ accumulation is the presence of functional ROS scavengers. Many aerobic respiring species such as *Escherichia coli* show a H₂O₂-producing phenotype when the genes encoding H₂O₂ scavengers are disrupted. Only a few LAB encode hemin-catalase and functionality of this latter enzyme requires an exogenous hemin source. LAB genomes encode a variety of thiol peroxidases such as glutathione, thioredoxin or alkylhydroperoxide reductases that were shown to be important factors for oxidative stress tolerance, acting in vivo as ROS-scavengers. Alternatively, LAB may use several physiologic adaptations to protect against oxidative damage. Intracellular accumulation of manganese, pyruvate, or glutathione can be effective in detoxification of ROS. In general, lactic acid bacteria use very few enzymes requiring iron as a cofactor or iron-sulfur clusters as a prosthetic group, which is thought to reduce the risk of damage due to Fenton chemistry.

There has been much speculation about the biologic role of peroxide production in a host-related context. The genes encoding pyruvate oxidase (SpxB) in *S. pneumoniae* and lactate oxidase LacD in *S. pyogenes* were identified as important virulence factors. For oral streptococci such as *S. mutans* and *S. gordonii*, SpxB was found to be essential for adhering to tooth surfaces, forming biofilms and for natural competence. H₂O₂ released by members of the microbiota can have a direct impact on host immune signaling cascades that depend on the oxidation state of thiols and tyrosine residues. In one case, DNA damage of host epithelial cells could be indirectly attributed to extracellular O₂− production by *E. faecalis*. In other studies, bacterial H₂O₂ production was associated with anti-inflammatory responses, for example *L. crispatus*-derived H₂O₂ was linked to increased PPAR-γ expression. A study on Type 1 diabetes in rats showed that *L. johnsonii* supplementation caused higher ileal H₂O₂ levels, and abolished activity of the immune modulator indoleamine 2,3-dioxygenase.

**The pursuit of the H₂O₂ source in *L. johnsonii***

Most studies showing a correlation between bacterial ROS and host effects use supplementation of ROS scavengers, antioxidants or even expression of ROS scavengers in bacteria. Unavoidably, this strategy also targets host-associated ROS production and may alter the redox state of essential enzymes in host immune signaling cascades. Our study demonstrates for the first time the in vivo biologic outcome when comparing the H₂O₂ negative Δnfr mutant of *L. johnsonii* to its wildtype counterpart. To identify the main H₂O₂ source in this species several genes that bared resemblance to H₂O₂ producing oxidases of other bacterial species were disrupted. Deletion mutants of genes annotated as lactate oxidase, pyruvate oxidase and/or NADH oxidase were constructed, but none resulted in

![Figure 1. Schematic representation of aerobic metabolic pathways in lactic acid bacteria involved in H₂O₂ production. LDH: lactate dehydrogenase, LOX: lactate oxidase, POX, pyruvate oxidase, ACK: acetate kinase, NOX: NADH oxidase, NFR: NADH flavin reductase.](image-url)
a H₂O₂ negative phenotype. An alternative attempt included the purification of the flavin-dependent NADH oxidation activity through ammonium sulfate precipitation, anion-exchange chromatography and size exclusion chromatography. LC-MS/MS analysis of a tryptic digest of the active fraction revealed the presence of peptides derived from an uncharacterized flavoprotein encoded by 2 highly similar genes. The disruption of the genetic locus encoding this flavoprotein resulted in a H₂O₂ negative mutant of L. johnsonii, whose H₂O₂ producing capacity could only be restored by plasmid-borne expression of both genes. The identity of these genes as a flavin reductase was confirmed by an independent study in the N6.2 strain of L. johnsonii. Further characterization showed that the 2 proteins formed a stable heterodimer harboring 2 flavin-binding pockets. The authors reported that for every mole of NADH, 0.56 mol of H₂O₂ was formed, and postulated that the reaction mechanism involved the formation of a semiquinone and a superoxide radical. A third gene with a PAS domain in a separate genetic locus that interacts with the dimerization process was identified, but this L. johnsonii strain was not genetically accessible and the effect of genetic disruption and/or overexpression of these genes could not be studied (personal communication).

The fitness advantage for L. johnsonii of carrying a flavin-dependent NADH oxidase encoded by nfr was experimentally determined by varying the oxygen availability. When growth kinetics of wildtype and Δnfr L. johnsonii were compared, the Δnfr mutant displayed higher sensitivity than wildtype to increasing oxygen availability. While no difference was observed between both strains in respect to the duration of the lag-phase under anaerobic conditions, in fully aerobic conditions, the lag-phase of the Δnfr mutant increased 2–3-fold. This fitness advantage of carrying a flavin-dependent NADH oxidase in a (micro-) aerobic environment has further been tested in competition experiments using serial batch cultivation. While under anaerobic conditions both strains still co-existed after 5 successive transfers, the wildtype strain took over at higher oxygen concentrations. In fully aerobic cultures the Δnfr mutant could not be identified by PCR at the end of the second transfer. This finding provides an interesting paradox: under fully aerated conditions, H₂O₂ production is the main cause of oxidative stress and growth stagnation. At the same time, this H₂O₂ producing capacity appears to confer aerotolerance. We speculate that this might be due to the oxygen scavenging activity of the NADH-flavin reductase. Especially in the host-associated context where H₂O₂ is not expected to accumulate to millimolar levels, scavenging oxygen using a flavin reductase may provide more oxidative stress resistance than allowing uncontrolled auto-oxidations.

**Protecting the host by redox regulation of virulence**

Our study indicates that the H₂O₂ producing capacity of lactic acid bacteria contributes to and even substitutes host ROS generation. Bacterial H₂O₂ release may support the host in repressing inflammatory responses, strengthening the epithelial barrier and accelerating restitution, but it is rather difficult to assess conclusively which of these biologic outcomes are solely dependent on H₂O₂ release and not on other secreted factors or cell surface components that can trigger host responses. However, we connected H₂O₂ directly to negative regulation of the locus of enterocyte effacement (LEE), thereby reducing C. rodentium virulence. LEE represents a cluster of virulence genes in certain E. coli strains (C. rodentium, EPEC, EHEC) that includes genes encoding the type III secretion system which are essential for injecting bacterial effectors into epithelial cells. In p22phox-deficient mice (p22ΔIEC), luminal and adherent C. rodentium collected at the peak of infection displayed markedly reduced expression of the LEE master regulator ler and LEE3 encoded effector escN. Similar results were obtained in wildtype mice supplemented with H₂O₂ producing wildtype L. johnsonii, but not with the Δnfr mutant, and in several in vitro settings. Control of LEE gene expression is complex, involving several positive and negative regulators and environmental determinants. In terms of diffusible environmental factors Branchu and coworkers identified recently the NO sensor nitrite-sensitive repressor (NsrR) as positive regulator of the LEE1, LEE4 and LEE5 operons, but a redox-sensitive regulator has not been described yet. To fully understand the antivirulence potential of all or only certain H₂O₂ producing lactobacilli as part of the overall colonization resistance to pathogens, more knowledge about enzymatic ROS sources in bacteria and their complex symbiosis between host and microbe will be essential.
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