Encounters with oxygen: Aerobic physiology and H₂O₂ production of Lactobacillus johnsonii

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Chapter 6

General discussion and outlook
This thesis addresses the consequences of oxygen and CO₂ exposure on the metabolism and transcriptome profile of the lactic acid bacterium *Lactobacillus johnsonii*. These two gaseous components were found to have a major influence on metabolism, growth, gene expression, yield, and viability. Here we discuss several questions that logically follow from our findings and we suggest future experiments that could bring us closer to answering these questions. Furthermore, we discuss the relevance of the research presented in this thesis for the interactions between *L. johnsonii* and the mucosal surfaces of host organisms.

**CO₂ growth dependency and cell death**

The growth requirement of *L. johnsonii* for CO₂ in the absence of oxygen is one of the clear novel findings in this thesis. Previously, other LAB, including *S. thermophilus* and a subset of *L. plantarum* and *L. lactis* strains (251, 252) were shown to depend on an exogenous C-1 source under certain conditions. Given the similarities in LAB metabolism, we expect that CO₂ dependency is a characteristic that is conserved among a variety of LAB.

One of the transcriptional responses to CO₂ depletion was the induction of the carbamoyl-phosphate pathway genes, reported in chapter 5. Regulation of this pathway was previously shown to play a role in governing CO₂ and pyrimidine metabolism in *L. plantarum* (252, 263, 269). Expression of the pyr-operon was also found to be controlled in several other LAB by a variety of conditions. For example, the pyr-operon was repressed during bile and acid stress of *L. rhamnosus* GG (281, 282), coculturing of *L. lactis* with *S. cerevisiae* led to substantial repression of the pyr-operon in *L. lactis* (283), and growth of *L. casei* in ‘soy milk’ compared to bovine milk induced the expression of this operon (284). We consider that many of these environmental changes also include a change in the availability of an environmental C-1 supply; bile solutions may contain bicarbonate, and low pH influences the CO₂/HCO₃⁻ equilibrium, while cocultivation with *S. cerevisiae* is expected to increase environmental CO₂ levels. Taken together, we consider it likely that many of the environmental conditions known to control the expression of the pyr-operon in LAB, may in fact be responses to the availability to an appropriate C-1 source for growth, which thereby plays an important role in metabolic control in these bacteria.

There are three distinctive characteristics of CO₂ dependency that we report here, that were not described before. Firstly, in contrast to other LAB, the CO₂ dependency of *L.
*Johnsonii* cannot be relieved by addition of pyrimidine nucleotides, arginine or aspartate. Even with addition of excessive amounts of these nucleotides and amino acids, the typical 2-fold difference in viability of *L. johnsonii* grown under pure nitrogen versus CO₂-rich conditions could not be complemented. One of the possible causes may be a dysfunctional pathway for incorporation of exogenous uracil. An indication for this is the high level of expression (amongst the 200 most highly expressed genes in *L. johnsonii*) of the *upp*-encoded uracil phosphoribosyltransferase in normal, CO₂-rich, conditions. This enzyme catalyzes the conversion of imported uracil to uracil monophosphate. Its high-level transcription would be expected in case *L. johnsonii* would depend on the continuous import of uracil for regular growth in MRS. However, preliminary studies with chemically defined medium from which uracil was omitted, did not reveal uracil auxotrophy in *L. johnsonii* in medium in which CO₂ is not actively removed. These findings imply that the carbamoyl-phosphate pathway is functional *in vivo*. The metabolic mechanism underlying CO₂ dependency in *L. johnsonii* deserves further exploration, since no supplementation strategy could be identified to complement this dependency by specific nutrients, which is clearly different from what has been found in various other LAB (see also chapter 5).

A second characteristic that was not previously identified in LAB, is the substantial induction of cell death that was associated with CO₂ depletion. Both in microcolonies (Anopore studies) and in liquid cultures, we observed that CO₂ depletion led to loss of membrane integrity (as demonstrated by propidium iodide staining) and abolished the capacity to grow (as demonstrated by viability plating). This is a remarkable observation, since the absence of components required for growth is normally bacteriostatic rather than bactericidal. Following our hypothesis on the relation between CO₂ and pyrimidine biosynthesis, we speculate that a depletion of the intracellular pyrimidine pool is the mechanism underlying the lethality of CO₂ depletion. Fatal DNA breaks due to disbalanced nucleotide pools is a well-known consequence of thymidine exhaustion in both prokaryotes and eukaryotes (275, 276), and such a mechanism may explain the CO₂-related cell death in our experiments.

This cell death is especially of relevance for the application of *L. johnsonii* in the food industry, in particular in its application as a probiotic. Survival of probiotic bacteria in industrial processes as well as in the final products can be considered as a prerequisite for the health beneficial effect they elicit in the consumer. It is an important notion that not only oxidative stress may lead to viability loss, but depletion of the available C-1 nutrients in the industrial and product environments may also be an important cause
for loss of bacterial (probiotic) viability.

A third surprising characteristic of CO₂ depletion was the relation to environmental oxygen, reported in chapter 4. In contrast to the expectations, bubbling a fermenter with N₂ + O₂ resulted in higher growth rates and biomass yields than bubbling with pure N₂. Similarly, L. johnsonii displayed significantly higher growth rates in the absence of acetate under aerobic conditions as compared to anaerobic conditions. This growth stimulatory effect of oxygen was shown to be dependent on pyruvate oxidase-mediated CO₂ and acetate production, since it could be abolished in a pox deletion derivative of L. johnsonii. These findings imply that oxygen, besides its deleterious consequences in terms of hydrogen peroxide production (see below), also has beneficial effects by reducing the fastidious growth requirements of L. johnsonii.

The role of NFR and NOX in aerotolerance of *Lactobacillus johnsonii*.

One of the most prominent differences between aerobic and anaerobic growth of L. johnsonii is the production of substantial amounts of H₂O₂. Accumulation of this H₂O₂ results in an approximately 10-fold lower biomass yield in the presence of oxygen, due to premature H₂O₂-induced growth stagnation. Continuous removal of H₂O₂ through the addition of exogenous catalase prevents aerobic growth stagnation.

In chapter 1 and 2 we discuss the role of two enzymes, NOX and NFR, in H₂O₂ production of L. johnsonii. Based on our findings we propose that NFR is constitutively expressed, whereas NOX is expected to complement NFR after longer-term oxygen exposure. This redundancy for H₂O₂ production capacity, in combination with the strongly increased oxygen sensitivity observed in a L. johnsonii derivative that lacks both NFR and NOX, implies that NADH oxidation, oxygen consumption and/or hydrogen peroxide production are important to sustain aerotolerance and to allow aerobic growth of L. johnsonii. However, our experimental approaches did not directly enable the identification of the precise role of H₂O₂ production in the overall aerobic physiology of L. johnsonii, and below we discuss some of the possible mechanistic explanations.

*Can H₂O₂:NADH oxidases confer aerotolerance by scavenging oxygen?*

One of the most striking observations on the role of the H₂O₂ producing enzymes NOX and NFR, is that they appear to be essential for aerotolerance of L. johnsonii: a deletion of the genetic loci that encode these enzymes resulted in a strain that
displayed significantly lower growth rate and final OD under aerobic conditions. This finding provides an interesting paradox; \( \text{H}_2\text{O}_2 \) production is the main source of growth inhibiting stress under aerobic conditions, but at the same time appears to be essential for growth under these conditions.

Preliminary experiments comparing oxygen consumption rates in wild type \textit{L. johnsonii} and its \( \Delta \text{nfr} \) and \( \Delta \text{nfr} \Delta \text{nox} \) derivatives, establish that \textit{nfr} and \textit{nox} encode the main oxygen scavenging capacity in \textit{L. johnsonii}, since the deletion of both genes led to a complete elimination of oxygen consumption. Based on these results and the other findings presented in this thesis, we postulate that NFR and NOX may contribute to aerotolerance by scavenging oxygen. In this proposition, we consider the possibility that molecular oxygen and possibly superoxide derivatives resulting from the spontaneous autoxidation of cellular components, may under certain conditions be more damaging than \( \text{H}_2\text{O}_2 \). The most predominant damaging reaction in bacteria due to \( \text{H}_2\text{O}_2 \) is the result of Fenton chemistry, demonstrated by the effectivity of iron chelators to reduce \( \text{H}_2\text{O}_2 \)-induced cell death (131, 157, 285). \textit{L. johnsonii} has a remarkably low number of enzymes that are predicted to contain an iron-sulfur cluster (3 vs 145 in \textit{E. coli}). If \textit{L. johnsonii} maintains very low intracellular iron pools, similar to other LAB (151, 286), hydroxyl formation through Fenton chemistry would be rare. Thereby, the cellular make-up of \textit{L. johnsonii} would render it intrinsically tolerant against low-levels of \( \text{H}_2\text{O}_2 \).

Moreover, we assume that in the natural habitat in which this type of lactobacilli are encountered, they are only transiently exposed to limited amounts of oxygen. Oxygen is thought to freely diffuse over the bacterial membrane, driven by the intra- and extra-cellular concentration difference. Once inside the cell, oxygen could react with cellular components such as flavins, resulting in superoxide production and launching a cascade of damage inducing reactions. However, superoxide formation and its detrimental consequences could be prevented by the effective scavenging of oxygen by NADH oxidases that convert it into \( \text{H}_2\text{O}_2 \). The small, uncharged \( \text{H}_2\text{O}_2 \) molecule can freely diffuse out of the cell as long as the extracellular \( \text{H}_2\text{O}_2 \) levels remain low (287). In a crowded environment such as the intestinal microbiota, where \textit{L. johnsonii} co-occurs with numerous catalase and peroxidase producing bacteria, one could plausibly assume that the released \( \text{H}_2\text{O}_2 \) would be quickly scavenged and is unlikely to accumulate to substantial levels.

The argumentation raised above could explain how in certain environments \( \text{H}_2\text{O}_2 \)-producing NADH oxidation could contribute to aerotolerance, by the prevention of
superoxide mediated damage, but at the same time preventing the excessive accumulation of \( \text{H}_2\text{O}_2 \) by exploiting the ecosystem’s \( \text{H}_2\text{O}_2 \) defusing capacity. Consequently, the absence of elaborate oxidative stress defense mechanisms in \( L. \text{johnsonii} \) may be a result of its adaptation to its natural habitat, where it expresses only a minimal defense against the limited oxygen exposure, and exploits its environment for effective detoxification of the \( \text{H}_2\text{O}_2 \) it produces, which is relatively nontoxic to \( L. \text{johnsonii} \) anyway.

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The notion that LAB use oxidases to scavenge oxygen from its environment is supported by two studies. Rezaiki \textit{et al.} showed that \( L. \text{lactis} \) grown under respiratory-permissive condition in Erlenmeyer-flasks can effectively create an anaerobic environment, indicated by low GFP fluorescence and ceased \( \text{H}_2\text{O}_2 \) production (53). In addition, Gibson \textit{et al.} demonstrate that the deletion of a protein identified as a water-forming NOX in \( S. \text{pyogenes} \) resulted in a \( \text{H}_2\text{O}_2 \) producing strain that displayed a severe aerobic growth defect. One of the possible explanations of this observation is that the protein that was annotated as NOX is in fact an NADH peroxidase, since it is difficult to distinguish between these enzymes (see above; Table S1.1). However, if the enzyme was correctly identified as an NADH oxidase, it could scavenge oxygen to a level that is sufficient to prevent \( \text{H}_2\text{O}_2 \) production via the aerobic lactate utilization pathway (288).

We propose to explore this hypothesis by the following set of experiments:

- a careful assessment of the toxicity of the different reactive oxygen species, for instance by engineering superoxide dismutase and catalase expressing variants of \( L. \text{johnsonii} \).
- an analysis of the intracellular iron pools, or the total iron content compared to species that do express ROS-scavenging enzymes. This could provide support for the hypothesis that \( L. \text{johnsonii} \) can withstand low \( \text{H}_2\text{O}_2 \) levels due to its intrinsic resistance against \( \text{H}_2\text{O}_2 \) damage.
• quantification of superoxide production in cultures and cell free extracts of *L. johnsonii* that lack both NOX and NFR (*nox, nfr* deletion derivative) could provide support for the theory that oxygen scavenging by NOX and NFR prevents superoxide production.

Interestingly, preliminary experiments with the introduction of plasmid borne expression of a SOD gene from *L. paracasei* in wildtype *L. johnsonii* and its Δnfr and Δnfr Δnox derivatives, indicated that SOD expression recovers H₂O₂ production in the Δnfr Δnox background, indicating that these cells contain superoxide radicals that act as substrate in the SOD catalized reaction leading to H₂O₂ production. Further experiments should include a comparison of aerotolerance levels in these strains. In case SOD expressions indeed improves aerotolerance in the Δnfr Δnox mutant, this would substantiate the proposed primary role of the H₂O₂ forming NADH oxydases in oxygen scavenging to prevent the formation of the more detrimental superoxide.

**Perspectives: the relevance of ROS in host/microbiota interactions**

The experiments that are presented in this thesis observe *L. johnsonii* in a relatively unnatural single-species environment which provides useful insights in the metabolism and physiology of *L. johnsonii*, but neglects all metabolic and physiological effects in complex microbial communities associated with mucosal surfaces that can be considered as its natural habitat. Here, we would like to take a broader perspective on bacterial H₂O₂ production and discuss how it may affect host/microbe interactions.

**Role of ROS in host defense and signaling**

Reactive oxygen species have a central role in the non-specific innate immune response. Pathogenic bacteria are engulfed in phagocytic vesicles of dedicated immune cells such as macrophages and neutrophils. Activity of phagocyte-NADPH oxidases (Nox2) generates high levels of superoxide, referred to as respiratory or oxidative burst. The high levels of superoxide results in the formation of secondary ROS such as H₂O₂ and hypochlorous acid (HOCL) collectively leading to the death of the phagocytized bacterial cell.

Besides this dedicated anti-bacterial use of ROS, H₂O₂ produced in non-phagocytic tissue by NOX (1,3,5) and DOUX (1,2) plays a role as signaling molecules in a variety of pathways. In many of these processes H₂O₂ does not transduce the signal directly but oxidizes thiol
peroxidases, thioredoxin or glutathione that function as secondary messengers. The cysteine residues of many regulatory enzymes, such as tyrosine phosphatases, protein kinase phosphatases, NF-κB and ubiquitins are sensitive to oxidation by these thiols and have been reported to be modulated by such redox signaling. Additionally, H$_2$O$_2$ produced by epithelial NOX can also interfere with signaling processes in pathogenic bacteria such as *Campylobacter jejuni*, where the oxidation of a tyrosine kinase prevents capsule formation, which is an important virulence factor (289).

NOX-related ROS production serves a variety of roles: it can act directly antibacterial, and simultaneously recruit an immune response by activating inflammasome, cytokines and prostaglandins (290). Dysregulation of this process can result in excessively high levels of ROS production and can induce hyperinflammation, which is a typical characteristic encountered in Inflammatory bowel disease (IBD). Hyperinflammation cascades induce mucosal tissue morphological destruction, including the formation of lesions, ulcerations and fibrosis (291). Interestingly, the oral administration of LAB that overexpress superoxide dismutase or catalase has been shown to attenuate disease symptoms in experimental animal models for IBD, like the chemically induced rodent (mouse or rat) colitis models (138, 292, 293).

At the same time, ROS-producing NOX, provides negative feedback on this system and plays an important role in suppressing the inflammatory cascades in inflamed tissue to recover its homeostatic, non-inflamed status. A defect in the Nox2 causes chronic granulomatous disease in humans, which is not only characterised by recurrent infections, but also by hyperinflammation. Similarly, Nox2 expression was found to play an essential role in the prevention of insulin resistance and diet-induced obesity in mice. These apparently dualistic roles of ROS indicate that its effect is highly dependent on its temporal, spatial and quantitative production pattern (294).

### Role of ROS in host/microbiota interactions

The epithelial cells that line the digestive tract are continuously exposed to enormous amounts of bacteria: ranging from $\sim 10^3$/g in the upper parts of the small intestine to $\sim 10^{12}$/gram in the colon. It is of utmost importance that immune responses are balanced and appropriately eradicate pathogenic and invading bacteria, whereas they should not respond excessively to the bulk of commensal bacteria in the gut.

An intriguing question is how H$_2$O$_2$ produced by bacteria may impact gut homeostasis and host signal transduction. Essential to bacterial H$_2$O$_2$ to emerge and affect host
immune responses is the presence of sufficient oxygen in the GI-tract. Studies on the oxygen content demonstrate that the majority of the gut volume is anaerobic, due to continuous scavenging of oxygen by the microbiota. Oxygen is continuously leaking in from epithelial tissue, creating a very steep gradient from the mucosal surface to the lumen (40, 41). Another gradient appears to be present in which the proximal regions of the small intestine (duodenum and jejunum) are considered to contain higher levels of oxygen compared to the strictly anaerobic colon (196). Oxygen availability in the micro-environments of the intestinal tract may be one of the dominant drivers of the spatial distribution of microbial specialists, in which microaerophilic microbes are generally found in samples taken close to the mucosal surfaces whereas the obligate anaerobic species are mostly found in the more anaerobic environments of the lumen and the colon (41, 295, 296). There are several studies detailing how intestinal oxygen levels impact bacterial metabolism, including the modulation of pathogenicity factors of Shigella (41), respiration of E. coli (297) and thiol/flavin export by Faecalibacterium prausnitzii (298). We consider that aerotolerance is an important factor for bacteria to reside in close proximity of epithelial surfaces, which can be considered as an important driver for direct modulation of host immune responses.

In such environments with fluctuating oxygen levels, ROS produced by LAB, could potentially have an impact on health and disease in the host. In in vitro cell culture systems it was shown that superoxide produced by E. faecalis leads to host-cell lipid oxidation generating the reactive compound 4-hydroxy-2-nonenal which induces DNA damage (62, 299). Also, H$_2$O$_2$-producing streptococci, such as S. pyogenes, S. mutans and S. pneumoniae are associated with disease in humans. S. pneumoniae is regularly found amongst the upper-respiratory tract microbiota of healthy individuals, but can cause bacteremia, meningitis and pneumonia in immunocompromised individuals. S. pyogenes can show a similar transformation from harmless commensal to lethal pathogen, whereas S. mutans and S. gordoni are mostly associated with dental caries. As we described in chapter 1, H$_2$O$_2$ production in these species is mostly catalyzed by the lactate and pyruvate oxidation pathways (LOX, POX, ACK). Importantly, expression of the pyruvate oxidase encoding spxB gene has been identified as an important factor for virulence of S. pneumoniae (88, 90, 300-302). Similarly, SpxB and H$_2$O$_2$ production was also correlated with the ability of oral streptococci to adhere to the tooth surface and release DNA, which is an important factor for biofilm formation and natural competence (85, 303-306), although the exact mechanism behind these correlations remains unresolved. Bacterial H$_2$O$_2$ production could play a role in pathogenesis on basis
of its cytotoxic effect on epithelial tissue, but at the same time the bacterial production
pathways are of importance for their aerotolerance and oxidative stress resistance. Other
studies indicate that modulation of characteristics like aerobic respiration or expression
of ROS-scavenging enzymes are also considered as virulence factors, while they also
contribute to survival of the bacterial cell (57, 116, 117). Thereby, it becomes difficult
to distinguish between a direct role in the virulence cascade and the ability to persist in
the host environment. Obviously, the latter capacity is a prerequisite to allow bacteria
to exert their detrimental and pathogenic effects, creating an intrinsic dilemma in the
design of adequate experiments to discriminate between direct, and persistence-driven
effects on pathogenesis.

In many of the aforementioned cases, bacterial ROS production is associated with
adverse health outcomes. However, there are several reports indicating that bacterial
H₂O₂ may also have a beneficial, anti-inflammatory effect. One study, by Voltan et
al., shows that L. crispatus derived H₂O₂ induces PPAR-γ expression in epithelial cell
cultures. Such increased PPAR-γ expression was previously associated with improved
gut homeostasis and reduced severity of colitis (201), through its capacity to decrease
NF-κB expression and thereby suppress inflammatory cascades. Administration of
H₂O₂-producing L. crispatus in mice, could substantially reduce severity of (chemically
induced) colitis, whereas a low hydrogen-peroxide producing (spontaneous) mutant
of L. crispatus did not show such an effect. This study illustrated in multiple ways that
H₂O₂ is essential for PPAR-regulation. However, since the spontaneous low-level-H₂O₂
producing mutants failed to adhere to the epithelial cells while the wild-type strain
adhered effectively, the prerequisite of close proximity of the bacteria and the epithelia
may have been a strong confounder in this study. Thereby, secondary mechanisms to
explain the differences between the two bacterial strain that are independent of their
H₂O₂ production capacity can not be excluded to play a role in the observed protection
against colitis (50).

Another study described the effect of L. johnsonii on rats that are genetically predisposed
to develop type 1 diabetes (T1D), which to a certain extent could also be linked to the
bacterial capacity to produce H₂O₂. The L. johnsonii strain used in this study, strain
N6.2 was previously found to be overrepresented in the microbiota of T1D-susceptible
rats that did not develop diabetes (307). Administration of this isolate to the rats was
shown to increase a variety of mucosal proteins associated with gut barrier function
and decrease the inflammatory cytokine IFNγ. Furthermore, it decreased the oxidative
stress response and the overall incidence of T1D in this rat model, while these effects
were not seen with another *Lactobacillus* strain (of the species *L. reuteri*) that also negatively correlated with type 1 diabetes symptoms (308). Interestingly, *L. johnsonii* administration was shown to inhibit the expression of indoleamine 2,3-dioxygenase (IDO) in host ileal mucosa. IDO is primarily involved in tryptohan catabolism, but also plays a central role in inflammation cascades, and its activity has been associated with several adverse health outcomes (309). *L. johnsonii* administration was shown to lead to higher ileal H$_2$O$_2$ levels, and in *in vitro* cell cultures it could be shown that H$_2$O$_2$ (produced by *L. johnsonii*) abolished IDO activity. Taken together these results create a tentative connection between the IDO inhibition and the H$_2$O$_2$ producing capacity of this bacterium *in vivo*, although it can not be excluded that the elevated ileal H$_2$O$_2$ levels were (in part) host-derived, and thus may not have depended (exclusively) on the production by *L. johnsonii* (203). Nevertheless, this study provides a strong indication that bacterially produced H$_2$O$_2$ could modulate IDO expression *in vivo*, and it would be very interesting to evaluate the effect of the *L. johnsonii* mutants described in this thesis that are unable to produce H$_2$O$_2$ in this rat model.

Collectively, these studies indicate that H$_2$O$_2$ may act as a bacterial effector molecule that can evoke immune modulations in the host. We hypothesize that analogous to host-related H$_2$O$_2$ production, the effect of bacterial H$_2$O$_2$ depends on its temporal, spatial and quantitative release in the GI-tract. Intestinal conditions that stimulate high level production of ROS by bacteria could potentially induce epithelial and mucosal damage, whereas low-level production of ROS by bacteria like *L. johnsonii* may successfully suppress excessive host immune responses by modulating the expression of central metabolic regulatory nodes, such as PPAR-γ and IDO that have established roles in metabolism-immune cross talk.

*Relevance of bacterial H$_2$O$_2$ in the vaginal microbiota*

Compared to the oral and intestinal microbiota, where ROS-producing bacteria form only a small subset of the overall community, the vaginal microbiota stands out by its high abundance of such bacteria. The vaginal microbiota of the vast majority of women in the reproductive age is dominated by one (or more) of four different species (*L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners*) belonging to the *L. acidophilus* group, of which at least three (*L. gasseri*, *jensenii* and *crispatus*) can produce substantial amounts of H$_2$O$_2$. Although colonization patterns vary as a function of the menstrual cycle, health and disease and are influenced by sexual intercourse and pregnancy, all major studies that have analyzed the constituents of the vaginal microbiota identify this
lactobacilli dominated ecosystem in healthy women (310-313). In addition, the absence of lactobacilli in this ecosystem is often accompanied by overgrowth of a collection of opportunistic species such as Gardnerella vaginalis and Atopobium vaginae (314-316). This microbial disbalance is referred to as bacterial vaginosis (BV), which affects approximately 10-20% of European women (317). Women with BV-type microbiota are also at a significant higher risk to acquire sexually transmitted disease, and increased risk of womb infections that can elicit preterm births (199, 200, 318, 319).

The lactobacilli-dominated microbiota of the vagina provide an intriguing contrast to the mucosal surfaces of the digestive tract that are colonized by tens to hundreds of different species, none of which is really dominating the ecosystem. Conversely, the vaginal environment is colonized by only a handful of dominant species with very similar features, including their capacity for H₂O₂ production (94, 187, 320). A long-standing hypothesis states that bacterial H₂O₂ production in the vaginal niche is the molecular trait by which lactobacilli prevent the colonization of other organisms (319, 321). However, this hypothesis is debated, and as an alternative explanation it has been proposed that the high levels of lactic acid in combination with a low pH may be more effective in creating colonization resistance. In addition, the oxygen availability, as well as the stability of H₂O₂ in the vaginal environment have been contested (312, 322). The precise role of bacterial H₂O₂ in vaginal homeostasis remains to be determined. The identification and mutation of the H₂O₂ producing enzymes in a species closely related to vaginal lactobacilli, that we presented in this thesis, may advance our understanding of this topic. We envision that a comparison between the ability of wildtype and non-H₂O₂ producing lactobacilli to prevent bacterial vaginosis in animal models could help understand the influence of this physiology on stability of the vaginal microbiota.

Relevance of bacterial H₂O₂ in the neonatal gut

The composition of the vaginal microbiota is not only related to health of women, but may also contribute to the health of an infant after vaginal delivery. Although it has been proposed that fetuses already acquire microbes prenatally (323), the predominant colonization of the infant gut takes place after birth. The vaginal bacteria of the birth canal may present an important part of microbial transfer between mother and infant. Comparison of microbial constituents of the neonatal gut following vaginal delivery with those found after caesarian delivery, revealed prominent differences and supported the idea that the vaginal (and/or fecal) microbes of the mother are amongst the pioneer colonizers of the neonatal gut after birth (16, 324, 325). Interestingly, these
lactobacilli containing early microbial colonization patterns are inversely correlated with the prevalence of necrotizing enterocolitis (NEC) in preterm infants (326, 327). The administration of probiotic lactobacilli has been shown to be effective in lowering the incidence of NEC (25).

Oxygen levels are generally assumed to show a significant drop during subsequent phases of colonization of the infant gut, which is reflected by the aerotolerance levels of the colonizing species. The first microbial groups that are found in the neonatal gut in the first 48h after birth are mostly aerotolerant, facultative or obligate aerobic species (16). Outgrowth of the strictly anaerobic groups, such as clostridia, faecalibacteria and sulfate-reducing bacteria, is typically found at later stages in the development of the microbiota (328). Although this dynamic colonization is influenced by many parameters, the establishment of the aforementioned oxygen gradients in the developing intestinal environment may play an important role in suppressing outgrowth of the more aerosensitive members of the microbiota. Some authors even suggest that oxygen consumption by the first aerobic colonizers is essential to pave the way for colonization of their anaerobic successors (40).

The observations described above raise the question whether H$_2$O$_2$ produced in the neonatal gut by the vaginal lactobacilli that were transferred during passage through the birth canal, may play a role in initiating or shaping the early immune system. This hypothesis is based on three assumptions: (1) that the absence of high numbers of bacteria lead to high oxygen levels in the GI-tract during the first hours after birth, (2) that mother-to-child microbial transfer leads to prominent colonization of the infant gut with H$_2$O$_2$-producing lactobacilli and (3) that bacterial H$_2$O$_2$ can modulate host-immune reactions. The third assumption is supported by observations described above that highlight the potential role of bacterially produced H$_2$O$_2$ in signaling to the mucosal via PPAR and IDO. Moreover, the capacity to scavenge oxygen could help the colonization of less aerotolerant species such as the bifidobacteria that are typically overrepresented in the intestinal microbiota of breastfed infants. However, one can also not rule out that the bacterially produced H$_2$O$_2$ may in certain cases also have adverse effects, since the presence of high intestinal oxygen levels and the absence of catalase expressing bacteria in the infant intestine may also cause ROS induced mucosal damage. Also here, small amounts may go a long way. The balance between ROS-production and ROS-scavenging are key in maintaining a healthy intestinal environment.
**Perspectives: how the findings in this thesis could help unravel the role of bacterial H₂O₂ in host/microbe interactions**

An important bottleneck in studying the effect of ROS in immune responses is the difficulty in teasing apart host and bacterially derived H₂O₂. Adequate negative controls that exclusively eliminate bacterial H₂O₂ from the interplay are currently not available. Especially the effects of ecosystem-derived catalase and SOD may scavenge both host and bacterially-derived ROS and may influence the colonization by specific microbial groups including the lactobacilli. A promising approach to resolve this issue was followed by Voltan et al., who used a spontaneous non-H₂O₂ producing isolate of *L. crispatus* (strain MU5) as a negative control. Unfortunately, this isolate was affected in various phenotypic traits besides its H₂O₂-producing capacity, including a non-aggregating phenotype and the inability to adhere to epithelial tissue cells (329). These deviations may be of critical importance for the *in vivo* effect that these bacteria could elicit in the intestinal tract of a host model, which was also supported by the finding that epithelial adherence was essential for the immunomodulatory capacity of *L. crispatus*.

The identification of the H₂O₂ producing enzymes in *L. johnsonii* and the availability of deficient mutants, opens novel avenues to further study the relevance of bacterial H₂O₂ in various host/microbiota interactions. The isogenic strains that are no longer able to produce H₂O₂, developed and studied in this thesis, NCC 9359 (*Δnfr*) and NCC 9360 (*Δnfr, Δnox*) could be instrumental in studies with epithelial tissue cultures and/or animal models. Moreover, similar mutants may more readily be constructed in other members of the *L. acidophilus* group, now that key-enzymes involved in H₂O₂ production (NFR and NOX) are identified in this group of bacteria. Inversely, H₂O₂-overproducing variants of *L. johnsonii* or other members of the *L. acidophilus* group could also be of interest. Although transformation with NFR encoding multi-copy plasmids (Chapter 2) did not lead to elevated H₂O₂ production by *L. johnsonii* in our experiments, it can not be excluded that alternative expression systems or specific (micro-aerobic) environments may allow the construction or exploitation of H₂O₂ overproducing strains in the same cell-based or animal models.