Encounters with oxygen: Aerobic physiology and H$_2$O$_2$ production of Lactobacillus johnsonii

Hertzberger, R.Y.

Citation for published version (APA):
Hertzberger, R. Y. (2014). Encounters with oxygen: Aerobic physiology and H$_2$O$_2$ production of Lactobacillus johnsonii.
Chapter 1
General introduction
Living on the edge: Reactive Oxygen Species production and scavenging in Lactic Acid Bacteria.
Abstract
Lactic acid bacteria (LAB) are generally classified as aerotolerant anaerobes. They grow relatively well in the presence of oxygen but cannot use oxygen as a terminal electron acceptor due to an incomplete electron transfer chain. Oxidases, such as cytochrome oxidase, NADH oxidase, lactate oxidase and pyruvate oxidase play a central role in the aerobic lifestyle of lactic acid bacteria, either protecting against oxidative stress (cytochrome oxidase, H$_2$O-forming NADH oxidase) or aggravating oxidative stress (pyruvate oxidase, lactate oxidase, H$_2$O$_2$-forming NADH oxidase). LAB employ several ROS-scavenging enzymes and molecules to provide resistance against oxidative stress. Here we give an overview of the literature that describes the role of oxidases and ROS-scavenging enzymes in aerobic metabolism and oxidative stress resistance of LAB.
Introduction

“There’s something new here—in the middle of the drop they are lively, going every which way.” Gently, precisely, a little aimlessly, he moved the specimen so that the edge of the drop was under his lens... “But here at the edge they’re not moving, they’re lying round stiff as pokers.” It was so with every specimen he looked at. “Air kills them,” he cried, and was sure he had made a great discovery.

– Paul de Kruif, Microbe Hunters, 1926 –

This is Paul de Kruif’s interpretation of what happened when Louis Pasteur first peered through his microscope at a specimen of rancid butter in 1860 (1). Previously, Antonie van Leeuwenhoek in the 17th century and Lazarro Spalanzani in the 18th century had already noted that some of these swarming animalcules live happily in the absence of air. Now Pasteur could add a new category to the list of lifestyles in the microbe world that were dictated by oxygen: air could also be toxic (2).

By now, we know that oxygen is not only toxic to these butyric acid producing clostridia that Pasteur was looking at, but that all forms of life that encounter oxygen need some strategy to deal with its hazardous byproducts. The risk is embedded in the chemical makeup of the oxygen molecule. The ground state of the oxygen molecule is the triplet state, which means that the single electron transfer to oxygen is thermodynamically highly favorable, but quantum-mechanically (spin) forbidden. As a consequence it is a relatively inert gas: the building blocks of the cell -lipids, amino acids, nucleotides- are only weak univalent electron donors so their autoxidation is a sluggish reaction. However, oxygen rapidly oxidizes other cellular compounds that are strong univalent electron donors, such as flavins and quinones (3). The products that are formed by these reaction are much stronger univalent electron acceptors than triplet oxygen and are known as reactive oxygen species (ROS, Figure 1.1). They can cause extensive damage through different pathways. Metal centers are especially vulnerable to superoxide. The enzymes that commonly rely on catalytically active [4Fe-4S]-clusters, such as fumarase and aconitase, are quickly inactivated by superoxide (4, 5). The release of free iron metals from damaged centers accelerates further ROS formation: hydrogen peroxide can interact with free ferric ions producing the highly reactive hydroxyl radical. If this so-called Fenton-reaction occurs in the proximity of DNA, it may cause DNA lesions and subsequent cell death. An excellent review on the specific damage that ROS may cause was written by James Imlay (6).
It is therefore not surprising that, on a planet with an atmosphere that consists for more than 20% of oxygen, there is an urgent need for many organisms to invest in mechanisms that eradicate reactive oxygen species from the cells (7). Even species that are dependent on a continuous supply of oxygen for aerobic respiration, require enzymes that continuously scavenge the toxic byproducts. The anti-oxidative arsenal consists of a variety of proteins specialized at this task, including catalase, and superoxide dismutase (SOD) that react with hydrogen peroxide and superoxide radicals, respectively (8).

The discoverers of superoxide dismutase, McCord and Fridovich (9, 10) hypothesized that ROS scavengers were the facilitators of an aerobic lifestyle, since their occurrence seemed to correlate with the aerotolerance of bacteria (11). Since then, it was shown that several bacteria that express these proteins indeed rely on their presence for aerotolerance (7, 12, 13). Yet, the physiology of other bacteria indicate that the proposed rule does not apply universally: obligate anaerobes such as the butyric acid producing clostridia that Pasteur had observed, express a variety of ROS-scavengers, including a functional superoxide dismutase (14, 15). Moreover – as McCord and Fridovich already observed for *Lactobacillus plantarum* - several lactic acid bacteria are aerotolerant but are devoid of a functional SOD or catalase. Such bacterial species challenge our understanding as to how micro-organisms may deal with the consequences of a life in the presence of oxygen.
1. Lactic acid bacteria

LAB are a phylogenetically diverse group of Gram-positive, non-sporulating, rod- or coccoid shaped bacteria that have a fermentative metabolism with lactic acid as a major end product. The intimate relationship between LAB and humans is related to the prominent role of these bacteria in food fermentations. They contribute to the taste, texture and shelf life of a wide variety of fermented food products including yoghurt, cheese, butter milk, sour cream, pickles, olives, sauerkraut, sourdough and meat.

Besides their presence in these food products, LAB are associated with the mucosal surfaces of the human body. They are amongst the very first microbes that colonize newborns: the lactobacilli that inhabit the vaginal cavity of the mother are microbial pioneers in the neonatal gut (16). Further bacterial transmission from the mother to the infant during breastfeeding also mostly consists of LAB (17, 18). LAB, and in particular streptococci, constitute a considerable part of the upper oro-gastrointestinal tract microbiota (19, 20).

In the gastro-intestinal tract, specific LAB species and strains have been proposed to exert a positive effect on the health of the host. Administration of adequate amounts of lactobacilli and bifidobacteria in products designated as probiotics can reduce the severity of antibiotic-associated diarrhea (21, 22), infectious diarrhea (23) and Clostridium difficile–related diarrhea (24). Probiotics can be effective in the prophylactic treatment or necrotizing enterocolitis in preterm infants (25) and they have been proposed to be effective in reducing the risk of atopic dermatitis development in children (26).

Several characteristics of LAB were identified that may contribute to these probiotic effects. Firstly, probiotic LAB generally display substantial acid and bile resistance that enables them to survive the hostile environment of the stomach and the upper digestive tract (27). Secondly, they express proteins that allow competitively adherence to intestinal mucus (28), and thirdly, they can strengthen the mucosal barrier and/or immune function (29, 30).

2. LAB and oxygen

In terms of their classification with respect to oxygen, LAB are generally referred to as aerotolerant anaerobes. This classification reflects two commonly conserved characteristics in LAB: (i) due to the incompleteness of their electron transport chain, they are unable to use oxygen as a terminal electron acceptor (hence the term “anaerobes”), while (ii) oxygen is also not particularly damaging either, which is
illustrated by the relatively good growth of many LAB in aerobic environments (hence the term “aerotolerant”). This tolerance does not mean that LAB are unresponsive to environmental oxygen levels. Oxygen can have a profound effect on growth, metabolism and viability. The presence of oxygen can dictate which pathways are used for ATP generation and which fermentation metabolites are produced (31, 32). The enzymes involved in pyruvate dissipation are particularly influenced by exposure to oxygen (see Figure 1.2). For example, pyruvate formate lyase (PFL) is highly sensitive to oxygen (33) and as a consequence, formate production is completely abolished upon oxygen exposure. The pyruvate oxidase (POX) pathway depends on molecular oxygen as a substrate (34) and facilitates acetate production with additional generation of ATP from central metabolism. Furthermore, oxygen allows the utilization of lactate through the lactate oxidase (LOX) pathway and the regeneration of oxidized reducing equivalents catalyzed by NADH oxidase (NOX) (Figure 1.2)

Figure 1.2: Overview of pyruvate metabolism in LAB (for simplicity the pathways for acetoin/2,3-butanediol were omitted). LDH: lactate dehydrogenase, LOX: Lactate oxidase POX: pyruvate oxidase, PFL: pyruvate formate lyase, PDH: pyruvate dehydrogenase, PAT: phosphate acetyltransferase, ACK: acetate kinase, NOX: NADH oxidase)

Besides the additional metabolic reorientation that oxygen may elicit, the generation of hydrogen peroxide \( \text{H}_2\text{O}_2 \) and superoxide species in aerobic metabolism, for instance by NOX or POX activity, is a major source of intrinsic oxidative stress in LAB. Especially
in obligate homofermentative lactobacilli $\text{H}_2\text{O}_2$ can freely accumulate and may cause growth stagnation and cell death (35-38). At the same time, oxygen can also provide a benefit in terms of additional ATP generation through the POX-pathway, involving activity of POX and acetate kinase (ACK, see Figure 1.2). Accordingly, the degree of aerotolerance encountered in individual LAB species and strains shows considerable variation. Some species may lean more towards “aerotolerant”, such as species in the *Leuconostoc* genus that can reach approximately 2-fold higher cell densities in aerobic environments as compared to anaerobic environments (39). Other species may lean more towards “anaerobes”, such as *Lactobacillus delbrueckii* subsp *bulgaricus* that reaches approximately 2-fold reduced cell densities in aerobic conditions (37). Results presented in this thesis demonstrate that *Lactobacillus johnsonii* also belongs to this latter group.

In the environments where LAB are regularly found, they will frequently encounter molecular oxygen and its detrimental derivatives. The epithelial and mucosal linings of the mouth, vagina and intestine are considered to contain considerable levels of oxygen (40-45). Similarly, LAB used in the preparation of fermented food products are exposed to oxygen during processing, shelf life and consumption (35, 46, 47). This regular oxygen exposure has a considerable impact on their overall metabolism and physiology (48), on their interactions with other bacteria (49) and on their interactions with the host organism (40, 50).

In this literature review we explore the aerobic physiology of LAB and in particular their aerotolerance. We consider two aspects that are central for the ability of cells to retain viable and to proliferate in the presence of oxygen. Firstly, we will discuss aerobic metabolism in LAB, which is presumably mediated by the activities of four oxidases: cytochrome oxidase, POX, LOX and NOX. Secondly, we will discuss expression of ROS-scavenging enzymes and other common anti-oxidative strategies that

---

**Definitions used in this thesis:**

**ROS** Hydrogen peroxide, superoxide, hydroxyl radical. For simplicity, we restrain ourselves to these three compounds (Figure 1.1).

**Oxidative stress** Cellular damage (or the risk of cellular damage) caused by ROS.

**Oxidative stress resistance:** The ability to grow and/or retain viability in the presence of ROS-generating compounds (e.g. paraquat and plumbagin) or hydrogen peroxide.

**Aerotolerance:** The ability to grow and/or retain viability in the presence of oxygen.
confer resistance against oxidative stress to LAB. Taken together, these aspects provide insight into the different consequences of oxygen exposure for different LAB species.

3. Cytochrome oxidase and respiration

A conserved characteristic amongst LAB is their inability to constitute a functional electron transfer chain that uses molecular oxygen as a terminal electron acceptor. Nevertheless, the requirements for a rudimentary electron transfer chain are present in several LAB, such as NADH dehydrogenase that accepts electrons from reducing equivalents (51) and cytochrome oxidase to catalyze the final electron transfer to oxygen (52). However, with a few exceptions (53, 54) most LAB are unable to synthesize quinones, that are essential for electron shuttling between NADH dehydrogenase and cytochrome oxidase. Furthermore, all LAB lack the essential genes to constitute a complete synthesis pathway for heme (55), an essential and functional prosthetic group of cytochromes and cytochrome oxidases. Therefore, LAB cannot assemble cytochromes and cannot produce functional cytochrome oxidases (52). In several species a respiratory phenotype can be induced by supplementation of hemin, indicated by increased biomass levels, cytochrome synthesis, altered metabolite levels and proton pumping (56-58).

Several studies on the respiratory phenotype report a dramatic increase in survival in respiration-permissive environments. The viability loss that is witnessed in certain LAB during aerobic stationary phase can be prevented by the addition of hemin, as was shown in L. lactis (59, 60), L. plantarum and S. agalactiae (57). Moreover, L. plantarum displayed a considerably higher H$_2$O$_2$-tolerance in the presence of hemin (61).

Besides the production of additional ATP, hemin addition may also contribute to aerobic robustness through reducing ROS production. The respiratory chain is an important source of reactive oxygen species. Reduced quinone species can spontaneously react with oxygen, resulting in superoxide and H$_2$O$_2$ formation. Enterococcus faecalis is an exception amongst LAB since it is can synthesize demethylmenaquinones. Univalent oxidation of these quinones was shown to be the cause of extracellular superoxide production (62, 63). Activity of cytochrome oxidase through the addition of hemin abolished this superoxide generation. Such an effect of cytochrome oxidase activity on ROS-production was previously observed in Escherichia coli where a flux through the electron transfer chain (by restoring the function of a cytochrome oxidase) could reduce H$_2$O$_2$ production derived from autoxidation of the flavin cofactor of fumarate reductase (64). In L. lactis, aerobic respiration also abolished ROS-production (53), although here
the major source of ROS was found to be a H$_2$O forming NADH oxidase (48). We consider however that lowering the NADH/NAD$^+$ balance in *L. lactis* by shuttling electrons from NADH in an electron transfer chain, would also lower the flux through the H$_2$O forming NADH oxidase and would therefore contribute to lowering superoxide-formation.

These studies show that in several LAB, aerobic respiration can increase yield and robustness, and oxidative stress tolerance. Oxidative stress is reduced by diminishing autoxidations of respiratory intermediates and by generating additional ATP. In addition menaquinone and/or hemin has an impact on a variety of physiological aspects, including metabolite profile, pH and redox-state. All these aspects may play a role in the increased oxidative stress tolerance.

4. NADH oxidase

One of the consequences of lacking an intact electron transfer chain is that additional redox constraints arise. Glucose to lactate fermentation is redox-neutral: the NADH that is produced through the glycolytic production of pyruvate, is regenerated by the lactate dehydrogenase (LDH) reaction in an equimolar stoichiometry. Pyruvate conversion to acetic acid (through one of the three pathways: PDH, PFL, POX) requires the oxidation of NADH through a different pathway, either by the production of reduced metabolites such as ethanol or acetoin or through the use of molecular oxygen to regenerate NAD$^+$ for example through the activity of NOX (Figure 1.2; (65-67).

A second complication in the correct identification of NOX is that other flavoproteins that do not belong to the NOXs may catalyze NADH oxidation as a side activity. Fumarate reductase of *Escherichia coli* (64, 68) and dihydroorotate dehydrogenase of *B. bifidum* (69) are examples of proteins with a solvent-exposed flavin moiety that easily autoxidizes, leading to superoxide or H$_2$O$_2$ production. Furthermore, free flavins that are reduced by NADH flavin reductases may spontaneously react with molecular oxygen producing considerable amounts of ROS (6). Reduced flavins can provide an important source of H$_2$O$_2$ in lactobacilli, which is illustrated by the identification of a flavin reductase as the main contributor to H$_2$O$_2$ production by *L. johnsonii* (chapter 2 of this thesis). Two different NOX enzymes are expressed by LAB: one that catalyzes a four electron transfer producing mainly water and one that catalyzes a two electron transfer producing mainly H$_2$O$_2$. It is not trivial to distinguish between these two forms since LAB may also express an NADH peroxidase (NPR), which in combination with the activity of H$_2$O$_2$-producing NOX results in the same overall reaction as the one catalyzed by the
H₂O-producing NOX (32, 70) (Table 1.1). In some cases, such as in the eukaryotic DUOX enzyme (71) and bacterial alkyl hydroperoxide reductase (72), the two reactions could even be attributed to a single enzyme. DUOX H₂O₂ producing and scavenging (NOX and NPR activity) can even be attributed to a single enzyme. Nox-1, a 55 kDa protein requiring exogenous flavin for activity, in Streptococcus mutans displayed a high level of homology with the earlier identified ahpF gene in Salmonella typhimurium (73) that is part of an alkylhydroperoxide reductase (AHPR, encoded by ahpC and ahpF, see also below at 6b). This Nox-1 catalyzes H₂O₂ producing NADH oxidation but together with the upstream located ahpC gene, it forms an AHPR, which can reduce H₂O₂ as well as alkyl organic peroxides using NADH (74, 75). A similar feature and DUOX enzymes in eukaryotes (71)

Table 1.1: Enzymatic reactions involving reactive oxygen species generally found in LAB.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome bd oxidase (Q = menaquinone or ubiquinone)</td>
<td>2 QH₂ + O₂ + 4 H⁺,in → 2 H₂O + 2 H⁺,out + 2 Q</td>
</tr>
<tr>
<td>Pyruvate oxidase (cofactors, TPP, Mg²⁺, FAD)</td>
<td>pyruvate + O₂ + +Pᵢ → acetyl − P + H₂O₂ + CO₂</td>
</tr>
<tr>
<td>Lactate oxidase</td>
<td>lactate + O₂ + H⁺ → pyruvate + H₂O₂</td>
</tr>
<tr>
<td>NADH oxidase (H₂O₂-forming)</td>
<td>NADH + H⁺ + O₂ → NAD⁺ + H₂O₂</td>
</tr>
<tr>
<td>NADH oxidase (water-forming)</td>
<td>2 NADH + O₂ + 2H⁺ → 2 NAD + 2 H₂O</td>
</tr>
<tr>
<td>NADH flavin reductase (uses either FAD, FMN or riboflavin as substrate)</td>
<td>NADH + H⁺ + flavin → NAD⁺ + flavinH₂</td>
</tr>
<tr>
<td>Catalase (with either hemin or manganese in the active site)</td>
<td>flavinH₂ + O₂ → flavin + H₂O₂</td>
</tr>
<tr>
<td>Thiol-based peroxidase. R could be substituted for TRX, GT or AHP.</td>
<td>2H₂O₂ → 2 H₂O + O₂</td>
</tr>
</tbody>
</table>
Streptococcus mutans also encodes a 50 kDa enzyme Nox-2 that catalyzes the transfer of four electrons to oxygen, producing water. This Nox-2 appears to be essential for regenerating NAD$^+$, since a nox-2 deletion derivative of S. mutans displays hampered growth on mannitol, which is a substrate that generates additional NADH when it is catabolized to pyruvate. Moreover, this deletion mutant also redirected its carbon metabolism towards a higher production of lactate, which is more reduced than the acetate and CO2 combined (76). In other LAB, these type of metabolic effects of NOX are also observed. In L. sanfranciscensis (65, 77) and in L. lactis (66, 77), NOX was shown to exert direct control on the end-product of fermentation. L. lactis shifted from homolactic to mixed acid fermentation upon controlled overexpression of a H$_2$O-forming NOX when growing in the presence of oxygen (66, 74, 77).

These examples illustrate that redox balance (NADH/NAD$^+$ ratios) plays a pivotal role in the control of the pyruvate dissipating flux. Lactic acid bacteria may be forced towards lactate formation to sustain redox balance under anaerobic conditions, but can shift towards mixed acid fermentation under aerobic conditions through the alternative electron sink provided by the NOX enzyme activity, with the concomitant benefit of the additional ATP generated through the acetate formation pathway. This metabolic control of redox balance has been exploited in LAB, and in particular in L. lactis to redirect its metabolic flux towards higher production of flavour components like acetoin, diacetyl and acetaldehyde (66, 78).
5. Aerobic lactate utilization: pyruvate and lactate oxidase

POX plays a prominent role in the metabolism of several well-studied LAB. It catalyzes the oxidative decarboxylation of pyruvate in the presence of inorganic phosphate, releasing acetyl-phosphate, carbon dioxide and $H_2O_2$. The enzyme is a homotetramere that contains tightly bound flavin adenine nucleotide (FAD) and uses thiamine pyrophosphate (TPP) and a divalent metal ion as cofactors (79, 80).

POX is part of the aerobic lactate utilization pathway. Upon glucose exhaustion in the environment as a consequence of aerobic growth of *L. plantarum* of *L. lactis*, the accumulated lactate can be oxidized by the combined activity of an NAD-dependent LDH and POX (81, 82). The resulting acetyl-phosphate can support ATP production through acetate kinase. Deletion of two (*poxB* and *poxF*) of this apparently five-fold redundant function in *L. plantarum* WCFS1 completely abolished aerobic lactate utilization and acetate production in stationary phase, implying that POX (and not PFL or PDH) is the main acetate-producing reaction in this species under these conditions (83).

A similar aerobic lactate utilization pathway is found in *S. pneumoniae*, although in this organism the first step is catalyzed by LOX instead of LDH. *S. pneumoniae* (then referred to as *Pneumococcus*) Expression of the *lox* (LOX) and *pox* (POX) genes in several LAB is repressed in the presence of glucose through CCP-A mediated catabolite control (84-87). This does not appear to be the case in *S. pneumoniae* where the transcription of these genes (88) nor the reaction they catalyze (lactate utilization, acetate and $H_2O_2$ production) were influenced by environmental glucose levels (89).

Combined LOX and POX activity results in the accumulation of up to 1 mM of $H_2O_2$ during the stationary phase of growth in *S. pneumoniae* (90) and *S. pyogenes* (86). Notably, deletion of either the *pox* or *lox* gene completely abolished $H_2O_2$ production and prevented the dramatic viability loss during the aerobic stationary phase of growth in several LAB (83, 86, 87, 89-91).

An exception with respect to the role of POX and LOX in aerobic metabolism is encountered in the species belonging to the *L. acidophilus* group. The *pox*-gene is present in these species and an active protein could be purified from *L. delbrueckii* subsp *bulgaricus* (34). Furthermore, as we show in this thesis, *L. johnsonii* can produce sufficient acetate and CO$_2$ to satisfy its growth requirements, in a POX and oxygen dependent manner (chapter 4). However, *L. johnsonii* and *delbrueckii* subsp *bulgaricus* are strictly homofermentative and thus do not produce acetic acid under aerobic conditions, which allows only a limited flux through the POX-pathway.
6. ROS scavenging enzymes

Many of the above-mentioned enzymes, such as POX, LOX, NFR and NOX, catalyze the transfer of two electrons to oxygen, resulting in H$_2$O$_2$ formation. Consequently, aerobic growth of some LAB is accompanied by the accumulation of substantial amounts (>1 mM) of H$_2$O$_2$. In streptococci this H$_2$O$_2$ is predominantly produced by POX and LOX (85, 86, 89, 92). In other bacteria, such as the species belonging to the *L. acidophilus*-group, H$_2$O$_2$ is produced in a reaction involving NADH (37, 93, 94). In this thesis we report the identification of the enzyme involved in H$_2$O$_2$ production in *L. johnsonii* as an NFR and identified a NOX enzyme to contribute to H$_2$O$_2$ production after prolonged aeration (see chapter 2 and 3). Besides the direct involvement of these enzymes, H$_2$O$_2$ may also be generated in spontaneous oxidations of cellular components. Superoxide radicals that are generated during autoxidation of dimethylmenaquinone in *E. faecalis* (62) and NADH oxidase activity of *L. lactis* (48) can spontaneously be dismuted to form H$_2$O$_2$. Even in aerobic, respiring bacteria such as *E. coli*, the deletion of the main H$_2$O$_2$ scavenging enzymes catalase and AHPR leads to H$_2$O$_2$ accumulation, indicating that this is a universal characteristic of bacterial growth in oxygenated environments. (64, 95).

Besides the ability to use oxygen for ATP generation, e.g. in aerobic respiration or aerobic lactate utilization, the ability to scavenge the toxic byproducts of oxygen reactions (ROS) is an important factor which contributes to aerotolerance of cells. LAB employ a diverse range of mechanisms to protect against the oxygen radicals that are generated during aerobic metabolism. Below we discuss the enzymes that were shown to contribute to aerotolerance and oxidative stress resistance in LAB by scavenging ROS. An overview of the distribution of genes encoding these ROS-scavenging enzymes in LAB is presented in Table 1.2 and S1.1).
Table 1.2: Distribution of genes encoding antioxidative/ROS-scavenging mechanisms in LAB genomes as found by BlastP (cut-off values: minimal query coverage 60%, maximal e-value $10^{-10}$, minimal identical residues: 20%). The number of genes that fall within the selection criteria are between brackets. For gene annotations and ID see Supplementary table S1.1.

<table>
<thead>
<tr>
<th></th>
<th>HemCat(^1) (accession nr. P60355)</th>
<th>MnCat(^2) (accession nr. P60355)</th>
<th>TrxR(^3) (accession nr. P60355)</th>
<th>Tpx(^4) (accession nr. P60355)</th>
<th>GshR(^5) (accession nr. A1YAC0)</th>
<th>AhpC(^6) (accession nr. 066265)</th>
<th>NPR(^7) (accession nr. A1211)</th>
<th>MnSOD(^8) (accession nr. A16429)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum WCFS1</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>L. lactis subsp. cremoris MG1363</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>L. rhamnosus GG</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>L. casei BL23</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>E. faecalis V583</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>S. pyogenes HSC5</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>S. pneumoniae D39</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>S. thermophilus LMG 18311</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>S. mutans UA159</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>L. sanfranciscensis TMW 1,1304</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>L. delbrueckii subsp bulgaricus ATCC 11842</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>L. johnsonii NCC 533</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^1\) Hemin-dependent catalase (HemCat) from *E. faecalis* V583 (96)
\(^2\) Mn-catalase (MnCat), *L. plantarum* ATCC 14431 (188)
\(^3\) Thioredoxin reductase (TRXR) *L. plantarum* WCFS1 (113).
\(^4\) Thiol peroxidase (TPX) from *S. pneumoniae* D39 (116)
\(^5\) Glutathione reductase (GSHR) *L. sanfranciscensis* DSM20451 (122)
\(^6\) Alkyl hydroperoxide reductase (AHPD), *S. mutans* NC11 1723 (74)
\(^7\) NADH peroxidase (NPR) from *Enterococcus faecalis* V583 (117)
\(^8\) Manganese-superoxide dismutase (MnSOD) *L. lactis* MG1363 (48)
a. Catalase
LAB are generally referred to as being catalase-negative and indeed, catalase encoding genes are sparsely encountered in LAB genomes. Nevertheless, two different types of catalases are found in LAB. A hemin-dependent catalase is present in the genomes of certain strains of L. plantarum, L. sakei, L. casei and E. faecalis (96, 97). This gene can produce a functional H$_2$O$_2$ scavenging enzyme when cells reside in hemin-containing environments, thus providing H$_2$O$_2$ tolerance under specific conditions (98-100). An alternative catalase that is not depending on hemin supplementation was identified in L. plantarum strain ATCC 14431 (101, 102) and appeared to employ manganese ions in its reactive center (103). The gene encoding MnCAT (accession nr. P60355) is very rare in LAB, and only encountered in a few Pediococcus, Enterococcus and Lactobacillus strains. A L. plantarum strain in which this MnCAT was absent produced H$_2$O$_2$ during aerobic growth indicating that this enzyme is the main H$_2$O$_2$ scavenging activity for these species in hemin-depleted conditions (100).

b. Thiol-based peroxidase
Whereas catalases are quite scarce in LAB, genes for a second type of H$_2$O$_2$-scavenging enzyme, with an activity that revolves around a catalytically active cysteine residue, are abundantly present on LAB genomes. The nomenclature in literature is quite diverse and elsewhere this group may be referred to as peroxiredoxins, cysteine-based peroxidases, thiol-specific antioxidants or NPRs. Here we will refer to them as thiol-based peroxidases, following Mishra et al (7). We discuss them as a group, since they show significant similarity, both in gene sequence and in reaction mechanism (104).

Thiol-based peroxidases catalyze the reduction of H$_2$O$_2$ to water, or organic hydroperoxides to their corresponding alcohol, using NADH or NADPH as the electron donor. Different types of thiol-based peroxidases have been detected in LAB: glutathione/glutathione reductase (GSH/GSHR), thioredoxin/thioredoxin reductase (TRX/TRXR) and alkyl hydroperoxide/alkyl hydroperoxide reductase (AHP/AHPR encoded by ahpC and ahpF, respectively). The reaction mechanism consist of two steps: the smaller polypeptide (either TRX, GSH or AHP) has an active site with two (seleno-)cysteine residues, or in the case of GSH two molecules that each contain a single cysteine residue. One of the cysteine residues reacts with H$_2$O$_2$, leading to the formation of a sulfenic acid that subsequently reacts with the secondary cysteine residue forming a disulfide bond. A dedicated flavoprotein reductase (either TRXR, GSHR or AHPR) can subsequently reduce the disulfide bond. The active site of these reductases also contains cysteine
residues and employs NADH or NADPH as an electron donor (7, 105, 106). In the case of thiol peroxidase (TPX), the disulfide bond is reduced by a TRX, which itself is reduced by TRXR. Besides their role in oxidative stress tolerance, these peroxidases protect against a wide variety of environmental stresses that are associated with the formation of reactive oxygen species, such as temperature and low-pH stress (107, 108). This type of NADH related peroxidase activity was shown to be complementary to catalase activity in *E. coli* (109). However, an important difference is that thiol-based peroxidases require input of electrons from reducing equivalents, whereas catalase does not need a co-substrate besides H$_2$O$_2$. Moreover, thiol-based peroxidases are active at lower ROS concentrations as compared to catalase (7, 74). Below, we discuss the prevalence of four types of thiol-based peroxidases in LAB, (i) thioredoxin/thiol peroxidase, (ii) glutathione reductase, (iii) alkyl hydroperoxide reductase and (iv) NADH peroxidase (Table 1.2 and Table S1.1).

*Thioredoxin reductase / Thiol peroxidase*

The genes encoding TRX (*trxA*) and its corresponding TRXR (*trxB*) are ubiquitously present in LAB (Table 1.2). TRX is a short polypeptide (103-106 residues) with a conserved cysteine rich catalytic site (CXXC). It is present in all forms of life and in several species it is indispensable. In bacteria, it has been associated with a variety of processes, including gene regulation and signal transduction. TRX can also function as an electron acceptor or hydrogen donor in redox reactions and can contribute to ROS scavenging (110-112). The catalytic cysteine residues of TRX react either directly with H$_2$O$_2$ or indirectly with an oxidized thiol from a so-called peroxiredoxin such as TPX. Oxidized TRX can be reduced by a dedicated TRXR, which has a corresponding CXXC conserved catalytic site. LAB generally have multiple copies of both the TRX encoding *trxA* gene and the TRXR encoding *trxB* gene. TRXR is a member of a larger family of pyridine nucleotide-disulfide oxidoreductases that also include GSHR, AHPR and DLD (catalyzing the second reaction in the pyruvate dehydrogenase complex). There are similarities between the members of this group, which complicates correct annotation. This is possibly the reason why not all copies of genes annotated as TRXR have the CXXC-motif which is deemed essential for its activity (113) (Table S1.1).

In several LAB, the TRX/TRXR systems were shown to contribute to oxidative stress resistance and aerotolerance. Mutation analysis of the TRXR encoding *trxB* gene in *L. casei* subsp. *shirota*, in *L. plantarum* and in *L. lactis* showed that the TRX/TRXR system
is essential for tolerance against disulfide (diamide)- and $\text{H}_2\text{O}_2$-stress (113-115). TRX expression was associated with the induction and repression of a variety of stress proteins in \textit{L. plantarum} (113) and \textit{L. lactis} (115). The \textit{L. lactis} \textit{trxB1} gene that contains the canonical CXXC-motif and is predicted to encode a TRXR was not reported to play a role in aerotolerance (115). Nevertheless, the \textit{L. lactis} genome contains a second TRX homolog, annotated as an AHP (33% identity) and contains the CXXC motif, which may show TRXR activity when the primary TRXR was disrupted in \textit{L. lactis}.

Notably, a TPX that functions in conjunction with the TRX system has been shown to display NADPH peroxidase activity in \textit{S. pneumoniae} (116), and to contribute to oxidative stress tolerance in \textit{E. faecalis} (117). Thereby the TRX, TPX system acts as a direct NPR that is involved in aerotolerance. Moreover, this system has also been shown to play a role in gene regulation related to the oxidative stress response in \textit{S. pneumoniae} (116).

\textit{Glutathione reductase}

Almost all LAB lack the enzymatic machinery for GSH synthesis: the GSH synthetase encoding gene from \textit{E. coli} has no full-length homologs in the \textit{Streptococcus}, \textit{Lactobacillus} and \textit{Lactococcus} genera. However, many LAB can import exogenous GSH and maintain high intracellular levels (108, 118). Furthermore, many species encode the corresponding GSHR, to regenerate reduced GSH. In \textit{L. lactis} intracellular GSH was estimated to increase to 60 mM when GSH-rich ingredients such as yeast extract were present in the medium. GSH supplementation was correlated with $\text{H}_2\text{O}_2$ tolerance (119) and provided cross-protection against acid stress (120). Similarly, in several LAB the uptake and recycling of GSH is correlated with oxidative stress resistance (118, 121-123).

\textit{Alkyl hydroperoxide reductase}

In \textit{E. coli}, the AHPR was observed to be the “first line of defense” against $\text{H}_2\text{O}_2$. The system, encoded by \textit{ahpC} and \textit{ahpF}, scavenges $\text{H}_2\text{O}_2$ with high affinity and low-level saturation ($K_m$ of 5 $\mu$M). It can fully complement catalase deficiency in aerobically growing \textit{E. coli} (109). As mentioned before, AHPR belongs to the same protein family as TRXR, GSHR and DLD and the corresponding genes are ubiquitously present on LAB genomes. However, copies of the corresponding AHP-encoding \textit{ahpC} gene are not as prevalent in LAB, indicating that this system is only functional in a subset of LAB (Table 1.2).
AHPR was first identified in *S. mutans* as a H$_2$O$_2$-producing NOX (76, 124). However, the direct reaction with oxygen appeared to be a side-activity. *In vivo* its main electron acceptor was identified as AHP, whose cysteine residues can be oxidized by H$_2$O$_2$, indicating its function in ROS scavenging (74, 76, 105). Such a role was established in *E. faecalis*, where deletion derivatives of *ahpCF* displayed reduced growth and viability in the presence of exogenous H$_2$O$_2$ compared to the wild type (117). However, this role of AHPR is apparently not universally valid, since *ahpCF* deletion derivatives of *S. mutans* were not affected in terms of their sensitivity towards H$_2$O$_2$ as compared to the wild type strains (125). Besides these apparently contradictory studies, the role of AHPR in ROS tolerance in LAB has not been studied extensively, and its *in vivo* function remains to be further elucidated.

**NADH peroxidase**

NPR is unique in the class of thiol-based peroxidases since both the reaction between thiol and H$_2$O$_2$ as well as the NADH recycling of the oxidized thiol is catalyzed by a single enzyme. The NPR of *E. faecalis*, encoded by the *npr*-gene, is a flavoenzyme with a single active cysteine residue Cys$_{42}$ (126, 127) that is essential for oxidative stress resistance in this bacterium (117). The NPR shows a remarkable redundancy in LAB. *L. plantarum* even has 10 genes that show resemblance with high significance (BlastP cut-off values: maximum e-value 10$^{-10}$, minimum identical residues 20%, minimum query coverage 60%). 6 of these 10 genes are annotated as NOXs and 5 have the conserved active site cysteine residue. One of these NPR homologs (*noxV*, accession number F9UUC2), displayed H$_2$O-forming NOX activity but its capacity to convert H$_2$O$_2$ was not investigated (128). The high-level of sequence homology between NOX and NPR may suggest that this group of flavoproteins could display substrate promiscuity and thereby play a role in NADH-dependent conversion of both oxygen and H$_2$O$_2$, and possibly also other electron acceptors.

**c. Superoxide dismutase**

Superoxide radicals in cells are formed during the autoxidation of cellular components that are strong univalent electron donors, such as quinones and flavins. The one electron transfer reaction between oxygen and flavin leads to the formation of a so-called flavinsemiquinone and a superoxide molecule. The reactivity of the flavinsemiquinone with triplet oxygen and the reactivity of fully reduced flavin with superoxide leads to
autocatalytic superoxide formation through redox cycling (129, 130). The reactivity of the superoxide radical towards iron-sulfur clusters is an important factor in oxidative stress damage. Damage to iron-sulfur clusters leads to the inactivation of proteins with such clusters. The release of iron from damaged iron-sulfur clusters accelerates the generation of ROS through the Fenton reaction (68, 131)(see Figure 1.1).

Although two superoxide molecules can spontaneously dismutate to form $\text{H}_2\text{O}_2$, the catalysis of this reaction by SOD is in many species an important means to prevent oxidative stress. The presence of a functional SOD in several LAB species is well-established (132-137). Interestingly, the main source of superoxide generation in *L. lactis* was shown to be a supposedly water forming NOX, where micromolar amounts of $\text{H}_2\text{O}_2$ appeared to be exclusively produced via the combined activities of NOX and SOD (48).

Whereas SOD in other organisms either uses iron, manganese or copper ions in its catalytic site, only the manganese-form was encountered in LAB. Expression of SOD was shown to contribute to aerotolerance (132, 134) and to $\text{H}_2\text{O}_2$-stress tolerance (125, 138), indicating that superoxide is a central oxygen intermediate contributing to cellular damage during oxygen exposure.

7. Physiological adaptations to protect against oxidative stress

Despite the presence of several homologs of thioredoxin reductases, NPRs and glutathione reductases, no NADH-related $\text{H}_2\text{O}_2$ scavenging activity can be detected in the cell extract of the well-studied lactic acid bacteria *L. lactis* (48) and *L. plantarum* (139, 140). Analogously, cell extracts of *L. plantarum* lack SOD activity but the cells show remarkable resistance towards hyperbaric oxygen levels (141). These results indicate that apart from expression of enzymes dedicated to ROS scavenging, lactic acid bacteria employ physiological adaptations to protect against toxic oxygen derivatives. Here, we discuss how LAB utilize metal homeostasis, accumulation of pyruvate and thiol metabolism as a means to reduce ROS-induced damage.

a. *Intracellular manganese accumulation*

Apart from its role as a cofactor of catalase and SOD, manganese is accumulated to high intracellular levels in *L. plantarum* (>20 mM) and functions as a superoxide sink. LAB that show such high intracellular manganese levels (such as *L. plantarum* and *L.*
casei) are generally more resistant against superoxide-generating compounds such as plumbagin as compared to LAB that do not accumulate high levels of manganese (such as *L. lactis* and *L. acidophilus*) (141-143).

There are several clues as to how such high intracellular levels of manganese may contribute to superoxide quenching. Bicarbonate-complexed manganese can contribute to H$_2$O$_2$ scavenging, which could indirectly also reduce superoxide toxicity (144). The authors of the original paper describing the phenomenon in *L. plantarum* suggested that pyrophosphate-complexed manganese could directly react with superoxide in vivo (141). Conversely, manganese accumulation in *E. coli*, which occurs in mutants lacking the genes for catalase and peroxidase, was effective in protecting against oxidative stress by replacing the reactive Fe$^{3+}$ ions in metalloproteins and thereby reducing Fenton chemistry (145, 146).

Attempts were made to identify the responsible manganese transporters in different LAB. The product of the *mnt*-gene was identified as a functional Mn$^{2+}$ uptake system in *S. oligofermentans* (147) and *L. plantarum* strain ATCC 14917 (148, 149) but not in strain WCFS1 (150). A recent study reported that in *S. oligofermentans*, manganese import was regulated upon increasing H$_2$O$_2$ levels and *mntA* expression substantially contributed to oxidative stress tolerance (147). However, the mechanism remains poorly understood and apart from these studies, no new research has appeared creating a link between regulation of manganese homeostasis and oxidative stress in LAB.

*b. Regulation of intracellular iron levels*

Compared to regulation of intracellular manganese levels, controlling intracellular iron may be an even more fundamental physiological adaptation that underlies the aerotolerant nature of LAB. Where other micro-organisms compete with each other to obtain sufficient bioavailable iron sources, LAB seem to have sidestepped this rivalry. Although there are several exceptions (in *S. pneumoniae* (91) and in heme supplemented growth conditions (151)), the growth requirements of several *Lactobacillus* species for iron are nearly zero and the intracellular iron levels are very low (152-154). As was mentioned before, such intracellular unbound iron can engage in ROS formation through the Fenton reaction which accelerates the damage to cellular components.

These low intracellular iron levels may be a direct consequence of the relatively low numbers of iron sulfur binding gene products in LAB compared to other bacteria such as *E. coli*. Moreover, several metalloproteins in LAB, such as catalase and SOD, were
shown to function with manganese ions instead of ferrous ions (see for instance our previous discussion of catalase and SOD in LAB). Through this reduced iron-dependency of protein function, lactic acid bacteria may avoid the need to maintain high intracellular iron pools, and may thereby be less sensitive to the toxicity of H₂O₂.

Besides the lack of iron-sulfur clusters, LAB also lack copies of the genes encoding bacterioferritin and ferritin, which are the major bacterial iron-storage proteins. The third iron binding protein DPS, which is associated with preventing oxidative stress (155, 156), is present in many LAB and showed to sequester iron and prevent H₂O₂-induced cell death in streptococci (75, 157, 158). We consider that even with low dependency on iron for protein function, oxidative stress in certain LAB is still correlated with levels of intracellular unbound iron.

c. Pyruvate accumulation
The metabolic intermediate pyruvate is an important branching point in the metabolism of LAB. Besides its role as central intermediate, pyruvate can be an effective H₂O₂ scavenger since it reacts non-enzymatically with H₂O₂, producing CO₂ and acetate. The scavenging effect of pyruvate is clearly illustrated by the observation that exogenously provided pyruvate can protect against H₂O₂-killing in different types of cells (159-161). L. lactis is reported to accumulate substantial amounts of pyruvate (>90 mM) when grown at low growth rates (48, 162). This excretion of pyruvate was found to effectively reduce extracellular H₂O₂ levels and accompanying oxidative stress (48). It is not clear whether this results from enhanced production rates or from reduced dissipation rates due to stress-induced reduced function of metabolic enzymes (163).

d. Cystine metabolism
Cystine is a dimer of two cysteine molecules. The supplementation and uptake of cystine by a cystine-binding protein encoded by cyuC was shown to prevent H₂O₂ accumulation in L. reuteri BR11 (164). The intracellular conversion of cystine into smaller thiols such as H₂S is catalyzed by cystathionine-γ-lyase. The gene encoding cystathionine-γ-lyase (cgl) is located in the same operon as cyuC, which also encodes a predicted cystine ABC-transporter (122, 165-167). The aerotolerance and oxidative stress resistance that this operon confers to L. reuteri BR11 indicates that such thiol metabolism may be involved in alternative pathways of oxidative stress resistance in LAB.
8. ROS production by LAB

Of the abovementioned enzymes and physiological adaptations that are correlated to oxidative stress, only a few (SOD, catalase and AHPR) were shown to contribute to ROS scavenging and prevent the accumulation of the most stable ROS species \( \text{H}_2\text{O}_2 \). An analysis of the prevalence of these proteins in LAB leads to the conclusion that these proteins are absent in LAB like \textit{S. pneumoniae} and \textit{L. johnsonii}. These species only encode proteins such as TRXR, TPX and GSHR (Table 1.2), for which expression was found to be associated with oxidative stress resistance but \textit{in vivo} ROS-scavenging functionality was not proven. An indication that these proteins are not, or only moderately involved in \textit{in vivo} ROS scavenging is provided by the relatively high-level of \( \text{H}_2\text{O}_2 \) production during aerobic growth of LAB encoding these proteins.

Hydrogen peroxide production by species of the \textit{L. acidophilus} group and some streptococci presents an intriguing dilemma since the accumulating \( \text{H}_2\text{O}_2 \) leads to growth stagnation and cell death. One would assume that this creates a substantial evolutionary pressure for the acquisition and expression of genes encoding ROS-scavenging proteins. The expression of either catalase, SOD or AHPR appears as an almost zero-cost option which would allow these species to counteract the damaging side-effects of the presence of oxygen. Nevertheless, despite their lack of ROS scavenging capacities, that are considered essential for aerotolerance in other bacteria, these \( \text{H}_2\text{O}_2 \) producing species show remarkable aerotolerance, with an initial aerobic growth rate that is comparable to the anaerobic growth rate and growth impairment only occurring when endogenously produced \( \text{H}_2\text{O}_2 \) reaches millimolar levels.

9. Concluding remarks

In this literature review we have provided an overview of the different protein expression and physiological adaptations LAB use to deal with oxygenated environments. We find three overarching themes that play a role in aerotolerance and oxidative stress resistance of LAB.

1. The extent in which LAB can profit from the presence of oxygen. Hemin supplementation induces a respiratory phenotype in several LAB, which results in higher biomass levels and lower oxidative stress. Acetate production through the POX pathway can result in additional ATP generation. Furthermore, acetate and \( \text{CO}_2 \) production through the POX pathway could relieve growth dependencies of some LAB and thereby expand the environmental niches that
can be colonized by these species (chapter 4 this thesis).

2 The extent in which a cell generates toxic derivatives during oxygen exposure. Several oxidases such as NOX, POX, LOX and NFR generate ROS which cause oxidative stress. Aerobic lactate utilization in many LAB (through LDH/LOX and POX, facilitated by NAD+ recycling by NOX) can cause cell death due to concomitant H₂O₂ production.

3 The extent in which a cell is resistant against the toxic derivatives of oxygen (ROS). LAB express several ROS scavengers, such as thiol-based peroxidases, catalase and SOD which are effective in protecting against oxidative stress. Furthermore, regulation of metal homeostasis, such as intracellular accumulation of manganese, and decreasing iron dependency of enzyme functions, leads to a cellular physiology that is more oxidative stress-tolerant. The absence of ROS scavengers are correlated with a considerably lower aerobic biomass levels.

We conclude that these cellular mechanisms underlie the general anaerobic aerotolerant nature of LAB. The diversity in phenotypes with respect to oxygen that is encountered within this group of bacteria, can in part be explained by the expression of oxidases and oxygen-dependent pathways, by the presence or absence of ROS scavengers and by physiological adaptations.

10. Lactobacillus johnsonii

*Lactobacillus johnsonii* NCC 533 (previously referred to as *Lactobacillus acidophilus* La1) is a gram-positive, rod-shaped, non-sporulating, bacteriocin and exopolysaccharide-producing lactic acid bacterium, with a low G-C content (34.6%), belonging to the *Lactobacillus* genus of the *Firmicutes* phylum. Within the genomically diverse *Lactobacillus* genus, *L. johnsonii* is assigned to the *L. acidophilus* group on the basis of similarities in DNA and rRNA sequence (168). This group of bacteria has received extensive attention due to their proposed probiotic properties (169-171), their occurrence in the microbiota of the gastro-intestinal tract as well as those encountered in the oral and vaginal cavities in humans (94). Furthermore, species such as *L. delbruecki* subsp. *bulgaricus*, *L. kefirofaciens* and *L. helveticus* are prominently present in different starter cultures for the production of yoghurt, cheese and kefir. Importantly, to date, none of the species of the *L. acidophilus* group has been recognized as a potential pathogen in mammals.

There has been a great effort in the last two decades to improve taxonomical classification
of this group, especially in order to refine the analysis of the enormous amount of human intestine microbiota data that have appeared over the past decade and to support the characterization of novel candidate probiotic strains. Comparative genomics studies revealed a high similarity at DNA, rRNA, protein, as well as metabolic level between the species of the *L. acidophilus* group (168, 172-175). For many of the species belonging to this group of lactobacilli there are now multiple genome sequences available and the group is continuously expanding due to the identification and sequencing of novel strains and species, which are often isolated from the GI-tract of mammals.

*L. johnsonii* was the second species of the *Lactobacillus*-genus for which the complete genome sequence was published (176). The sequence indicates that this species has been subject to a process of reductive evolution, displaying a remarkable loss of genes (177, 178). *L. johnsonii* lacks the genes for the biosynthesis of numerous compounds, including secondary metabolites, amino acids, vitamins, and fatty acids. This is reflected in its fastidious growth requirements and its lack of metabolic versatility.

*L. johnsonii* has been of particular interest to the food industry for its health-supporting properties. It is commercially used as the probiotic ingredient in the LC-1 fermented-dairy products marketed by Nestlé. In this application context, several studies have specifically looked at features related to adherence to the gut epithelium (171), its activity against enterovirulent pathogens *in vitro* and *in vivo* (38, 179, 180) and its immunomodulatory properties (181-183). In addition, broader understanding of the physiology and metabolism of *L. johnsonii* has been of relevance (184-186) to improve its survival and robustness in the harsh conditions encountered in industry and in the upper-gastrointestinal tract. These metabolic characteristics, such as its fastidious growth requirements, may also impact the interactions of *L. johnsonii* with its host organism.

11. Outline of this thesis

*L. johnsonii* is, like other LAB, an aerotolerant anaerobe. Aerobic growth of this species is accompanied by the production of millimolar amounts of H$_2$O$_2$ (38), which in part results from the absence of several of the ROS-scavenging mechanisms present in other LAB (Table 1.2). This H$_2$O$_2$ production is an intriguing characteristic which *L. johnsonii* shares with several lactobacilli (94, 187) and streptococci (74, 85, 86). The enzymes involved in H$_2$O$_2$ production in *L. johnsonii* had previously not been elucidated.

One of the primary goals of this thesis was to study the molecular mechanisms underlying
H₂O₂ production in L. johnsonii, e.g. to identify and characterize the enzymes that catalyze the H₂O₂ producing reactions and decipher the role of H₂O₂ production in the aerotolerance of L. johnsonii. In more generic terms the thesis also intends to provide a more global understanding of molecular responses of L. johnsonii to the presence of molecular oxygen.

In the first two experimental chapters of this thesis, we partially unravel the H₂O₂ producing reactions and aerobic metabolism in Lactobacillus johnsonii. In chapter 2, we identify and characterize the main H₂O₂ producing enzyme in L. johnsonii, which belongs to a novel NADH flavin reductase (NFR) enzyme family. In chapter 3 the genome-wide transcriptional response of L. johnsonii to the presence of oxygen is studied, leading to identification of a secondary H₂O₂ producing enzyme, which belongs to the NADH oxidase enzymes and appears essential for aerotolerance in the absence of NFR.

The relationship between specific growth dependencies of L. johnsonii in the presence and absence of oxygen are subsequently studied in chapters 4 and 5. In chapter 4 we demonstrate that oxygen is not only detrimental for L. johnsonii, but can also relieve some of its growth dependencies. Endogenous acetate and CO₂ production through the oxygen-dependent POX-reaction overcomes the dependency of this bacterium on exogenous C1 and C2- sources. In chapter 5, the growth dependency of L. johnsonii for CO₂ is further characterized by analyzing the genome-wide transcriptional response to CO₂ depletion. These analyses indicate that especially the pyrimidine biosynthesis pathway is depending on a CO₂ supply.

In chapter 6 the experimental results are discussed in the light of our current knowledge of the physiology of these types of lactobacilli. In addition, we discuss the implications of O₂ and CO₂ metabolism by L. johnsonii and other lactobacilli in the context of their interaction with mammalian host organisms.
## Supplementary materials

**Table S1.1: Antioxidative/ROS-scavenging mechanisms in LAB genomes.** Cut-off values for BlastP: maximum e-value: $10^{-10}$, minimum identical 20%, minimum query coverage 60%.

<table>
<thead>
<tr>
<th></th>
<th>HemCat</th>
<th>TrXR (query) 1 TRXR</th>
<th>Tpx (accession number)</th>
<th>GshR (accession number)</th>
<th>AhpC (accession number)</th>
<th>NPR (query) 1 NPR</th>
<th>MnSOD (query)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em></td>
<td>HemCat ef_1597</td>
<td>2 ferredoxin NAD(P) reductase, lp_2585</td>
<td>TPX, lp_2323</td>
<td>1 GSHR, lp_1253</td>
<td>-</td>
<td>1 NPR, lp_2544</td>
<td></td>
</tr>
<tr>
<td>WCFS1</td>
<td>lp_3578</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris MG1363</td>
<td>-</td>
<td>1 TRXR, limg_1588</td>
<td>TPX, limg_0318</td>
<td>1 GSHR, limg_1702</td>
<td>AhpC, limg_0356</td>
<td>1 NOX, limg_0408</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 AHPR, limg_0357</td>
<td></td>
<td></td>
<td></td>
<td>2 NOX, limg_1770</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 TRXR, limg_0776</td>
<td></td>
<td></td>
<td></td>
<td>3 hypothetical limg_1249</td>
<td></td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>-</td>
<td>1 TRXR, lgg_00920</td>
<td>TPX, lgg_00728</td>
<td>1 GSHR, lgg_02615</td>
<td>AhpC, lgg_02490</td>
<td>4 PNDR, lgg_2331</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 TRXR, lgg_00810</td>
<td></td>
<td></td>
<td></td>
<td>5 DLD, lgg_0071</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> BL23</td>
<td>-</td>
<td>1 TRXR, lcabl_10620</td>
<td>TPX, lcabl_08080</td>
<td>1 GSHR, lcabl_23620</td>
<td>AhpC lcabl_26730</td>
<td>1 NPR, lcabl_04690</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 TRXR, lcabl_08900</td>
<td></td>
<td></td>
<td></td>
<td>2 NOX lcabl_02800</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 NOX lcabl_01750</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> V583</td>
<td>HemCat (query)</td>
<td>1 TRXR, ef_1338</td>
<td>AhpC/TSA family protein, ef_2932</td>
<td>1 GSHR, ef_3270</td>
<td>AhpC, ef_2739</td>
<td>1 NPR (query)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 TRXR, ef_2738</td>
<td></td>
<td>2 DLD, ef_1356</td>
<td></td>
<td>2 NOX, ef_1586</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 PNDR, ef_2899</td>
<td></td>
<td>3 DLD, ef_1661</td>
<td></td>
<td>3 coenzyme A disulfide reductase ef_2989</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 PNDR, ef_1932</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 DLD, ef_1356</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Gene Accessions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes H5C5</td>
<td>1 TRXR, i897_06810, 2 NDH, i897_08795, 3 ferredoxin NADP reductase, i897_03465, 4 DLD, i897_03940</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae D39</td>
<td>1 TRXR, spd_1287, 2 PND, spd_1393, 3 PND, spd_1415, 4 TRXR, spd_1417, 5 DLD, spd_1048</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. thermophilus LMG 18311</td>
<td>1 TRXR, stu_1650, 2 TRXR, stu_1417, 3 DLD, stu_1048, 4 TRXR, smu_463, 5 TRXR, smu_869, 6 DLD, smu_130, 7 DLD, smu_1424</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. mutans UA159</td>
<td>1 TRXR, smu_463, 2 AHPR, smu_765, 3 TRXR, smu_869, 4 DLD, smu_130, 5 DLD, smu_1424</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. sanfranciscensis</td>
<td>1 TRXR, isa_05170, 2 ferredoxin NADP reductase, isa_02530, 3 TPX (query), 4 Lipid hydroperoxide peroxidase, smu_924, 5 Hypoth., isa_05160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. delbrueckii subsp bulgaricus ATCC 11842</td>
<td>1 TRXR, ldb_0613, 2 TRXR, ldb_1586, 3 TPX, lj_0852, 4 hypoth., lj_0501</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. johnsonii NCC 533</td>
<td>1 TRXR, lj_0852, 2 hypoth., lj_0501, 3 TPX, lj_1153, 4 hypoth., lj_0042, 5 PND, ldb_0759, 6 PND, ldb_1586</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>1 TRXR, jw_0871, 2 AHPR, jw_0599, 3 TPX, jw_1317, 4 AHPC, jw_1106, 5 DLD, jw_0112, 6 1 MnSOD, jw_3879, 2 SOD (Fe), jw_1648</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Hemin-dependent catalase (HemCat) from E. faecalis, (96), 2 Thioredoxin reductase (TRXR) L. plantarum WCFS1 (113), 3 Thiol peroxidase (TPX) from S. pneumoniae D39 (116), 4 Glutathione reductase (GSHR) L. sanfranciscensis DSM 20451 (122), 5 Alkyl hydroperoxide reductase (AhpC), S. mutans NC1B2 11723 (74), 6 NADH peroxidase (NPR) from Enterococcus faecalis V583 (117), 7 Manganese-superoxide dismutase (MnSOD) L. lactis MG1363 (48), DLD = dihydrolipoamide dehydrogenase, PND = pyridine nucleotide-disulphide reductases/oxidoreductases, TPX = thiol peroxidase, GSHR = glutathione reductase, TRXR = thioredoxin reductase, NDH = NADH dehydrogenase, AHPR = alkyl hydroperoxide reductase.