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

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Citation for published version (APA):
Summary (for a scientific audience)

Lactic acid bacteria (LAB) are intimately entwined with human life. They ferment several key food products in our diets and they reside on the mucosal surfaces of the mouth, intestine and vagina. Administration of adequate amounts of certain LAB species has been shown to provide health benefits, such as attenuation of antibiotic-associated diarrhoea. *Lactobacillus johnsonii* is a LAB that is used in the food industry for such health-benefit, or probiotic effects. The functionality of *L. johnsonii* and other related LAB in the food industry and in the host-related environment are strongly dependent on environmental factors. Especially oxygen and carbon dioxide were found to have a major influence on metabolism, growth, gene expression, yield, and viability. This thesis addresses the consequences of exposure to these two gaseous components on *L. johnsonii*.

Chapter 1 contains a general overview of research literature on aerobic metabolism and oxidative stress of lactic acid bacteria. LAB are classified as aerotolerant anaerobes; although they commonly grow well in the presence of oxygen they do not show aerobic respiration unless a hemin source is added. Instead, oxygen is used for direct oxidation of various metabolic intermediates. Facultative heterolactic LAB produce acetate and CO$_2$ aerobically through the pyruvate oxidase and lactate oxidase pathway. These products are more oxidized than lactate and therefore additional oxidation of the reducing equivalent NADH is required. When exogenous hemin is added to the medium, NADH can be channelled through the electron transport chain. This respiratory growth is associated with higher yield, robustness and lower oxidative stress. Alternatively, water or H$_2$O$_2$-forming NADH oxidases can regenerate NAD$^+$. NADH oxidase is a central switch in the metabolism of heterolactic LAB. Activity of these oxidases and autoxidation of other cellular components results in the generation of reactive oxygen species, such as hydrogen peroxide and superoxide, which cause oxidative stress. We provide an overview of the anti-oxidative enzymes (ROS-scavengers) and physiological adaptation found in LAB to reduce oxidative stress.

A prominent characteristic that *L. johnsonii* shares with several other lactobacilli and streptococci is the accumulation of substantial amounts (>1 mM) of hydrogen peroxide in its environment. *L. johnsonii* lacks the key ROS scavenging enzymes, such as catalase, alkyl hydroperoxide reductase and superoxide dismutase. Hydrogen peroxide accumulation is the primary cause of oxidative stress in *L. johnsonii*. It induces a premature stationary phase and a ~10-fold lower biomass yield in the presence of
oxygen. Addition of catalase abolishes this growth stagnation. In chapter 2 and 3 we report on the identification and characterization of two proteins that contribute to hydrogen peroxide production of *L. johnsonii*. In chapter 2 we showed that \( \text{H}_2\text{O}_2 \) production is unrelated to expression of pyruvate, lactate and NADH oxidase. In cell extracts of *L. johnsonii* an enzymatic hydrogen peroxide forming activity could be detected upon addition of flavin and NADH (not NADPH). Partial purification of the unidentified enzyme displaying this activity showed that two small flavoproteins, *LJ*\_0548 and *LJ*\_0549, were overrepresented in the hydrogen peroxide forming fraction. Genetic disruption and overexpression confirmed that these proteins constitute an NADH flavin reductase (NFR). A deletion derivative of these two genes did not produce any hydrogen peroxide when exposed to oxygen, indicating that these enzymes catalyze the reaction that produces hydrogen peroxide in *L. johnsonii*.

However, after prolonged cultivation in the presence of oxygen, the NFR deletion derivative regained partial \( \text{H}_2\text{O}_2 \) producing capacity. In chapter 3 we report on the identification of a second \( \text{H}_2\text{O}_2 \) producing enzyme, an NADH oxidase (NOX), encoded by the *LJ*\_1254-1255 locus. Expression of this locus was 2.1-fold induced in the wildtype under aerobic growth conditions and 3.7-fold in the NFR-deletion derivative. Deficiency of this NOX activity did not impact \( \text{H}_2\text{O}_2 \) production of the wildtype, but completely abolished all \( \text{H}_2\text{O}_2 \) production in its NFR deficient derivative. Intriguingly, this mutant also showed hampered growth and lower biomass yield in the presence of oxygen, despite its \( \text{H}_2\text{O}_2 \)-negative phenotype. We conclude that the oxygen-induced NADH oxidase produces hydrogen peroxide in the absence of NADH flavin reductase, and may also contribute to hydrogen peroxide production during longer exposure to oxygen.

Oxygen is not in all cases detrimental for growth and viability of *L. johnsonii*. In the second part of this thesis we focus on the growth stagnation observed during growth under a \( \text{N}_2 \) atmosphere, observed both in liquid (sparged batch cultures) as on solid media (Anopore™ slides). The cause of this growth stagnation was shown to reflect a lack of \( \text{CO}_2 \) which *L. johnsonii* requires for growth. Two aspects of this observation were unexpected. Firstly, oxygen could fully relieve this \( \text{CO}_2 \) growth dependency, and secondly, lack of \( \text{CO}_2 \) apparently led to cell death of *L. johnsonii*. Especially the latter effect is unusual, since removal of essential nutrients generally only halts bacterial growth. We further study these two factors in chapter 4 and 5. In chapter 4, we showed that this oxygen relieve of \( \text{CO}_2 \) dependency also accounted for the acetate growth dependency: *L. johnsonii* cannot grow in an environment without acetate unless the culture is aerated. Both these effects of oxygen could be traced back to one
common denominator, which is the pyruvate oxidase reaction leading to both CO₂ and acetate production. Pyruvate oxidase could therefore allow *L. johnsonii* to overcome its acetate and CO₂ dependency in conditions that include oxygen exposure. A pyruvate oxidase deletion derivative confirmed this hypothesis as it rendered the organism both aerobically and anaerobically dependent on acetate and CO₂ supplementation. Our results demonstrate that certain growth requirements of *L. johnsonii* are not hardwired but depend on environmental factors.

In **chapter 5**, we attempted to further understand the metabolic requirement for CO₂ by analyzing genome-wide transcriptome changes in an anaerobically growing culture upon CO₂ depletion. We detected an extensive rearrangement of gene expression, including many transporters and regulators. Additionally, expression of the *pyr*-operon encoded pyrimidine biosynthesis pathway, also referred to as the carbamoyl-phosphate pathway, was strongly upregulated (up to 17-fold). In other LAB, this pathway has been associated with CO₂ dependency, which is required in the first enzymatic step of the pathway and generally growth stagnation due to CO₂ depletion could in these LAB be prevented by addition of sufficient levels of pyrimidines. However, in *L. johnsonii* such a relation between pyrimidine supplementation and CO₂ dependency was not observed. Supplementation of other compounds that have previously been associated with CO₂ growth dependency in LAB, such as arginine and aspartate also could not prevent CO₂ induced growth stagnation. We speculate that *L. johnsonii* is unable to incorporate exogenous pyrimidines and propose further experiments to test this preposition.

In **chapter 6**, we present a general discussion of the results in this thesis and place them in the environmental context of the intestinal and vaginal microbiota. One of the most prominent questions that remains is why NOX and NFR activity is required for aerotolerance. We propose that H₂O₂ production of *L. johnsonii* may be a means to scavenge oxygen in an physiologically attractive way, i.e. without spending much NADH which is ultimately required for lactate production. High levels of intracellular molecular oxygen and possible resulting superoxide formation could be more hazardous than hydrogen peroxide, which quickly diffuses out of the cell and is potentially scavenged by neighboring catalase-expressing bacteria or other environmental factors. We propose several experiments to study this hypothesis.

Lastly, we discuss how hydrogen peroxide production and aerotolerance could play a role in the interactions of *L. johnsonii* with a host organism. Reactive oxygen species play a central role in a wide variety of immune reactions. We discuss how bacterially-
derived superoxide and hydrogen peroxide could contribute to gut homeostasis through immune signaling. Especially in the neonatal intestine, which generally contains higher levels of oxygen, H$_2$O$_2$-producing lactobacilli, transferred from the mother to the infant during birth, could produce substantial levels of hydrogen peroxide, which may contribute to shaping the neonatal immune system. We propose how the NOX and/or NFR deletion derivatives that were constructed in the scope of this thesis, can be employed to testing the role of bacterial H$_2$O$_2$ in host/microbiome interactions.