



*Encounters with Oxygen. Aerobic Physiology and H₂O₂ Production of
Lactobacillus johnsonii*

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Summary (for a scientific audience)

Lactic acid bacteria (LAB) are an important accessory to 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 several health benefits, such as attenuation of diarrhoea. *Lactobacillus johnsonii* is an LAB that is used in the food industry for this so-called probiotic effect. Its metabolism, its adaptive potential and general growth characteristics are therefore interesting research topic. This thesis addresses the consequences of oxygen and CO₂ exposure on the metabolism and physiology of this bacterium. These two gaseous components were found to have a major influence on metabolism, growth, gene expression, yield, and viability.

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 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 CO₂ 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 H₂O₂-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 show that H₂O₂ production is unrelated to expression of pyruvate, lactate and NADH oxidase. In cell extracts of *L. johnsonii* an enzymatic hydrogen peroxide forming activity is detected upon addition of flavin and NADH (not NADPH). Partial purification of the unidentified enzyme displaying this activity shows that two small flavoproteins, *LJ_0548* and *LJ_0549*, are overrepresented in the hydrogen peroxide forming fraction. Genetic disruption and overexpression confirm that these proteins constitute an NADH flavin reductase (NFR). A deletion derivative of these two genes does not produce any hydrogen peroxide when exposed to oxygen, leading to the conclusion 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 regains partial H₂O₂ producing capacity. In **chapter 3** we report on the identification of a second H₂O₂ producing enzyme, an NADH oxidase (NOX), encoded by the *LJ_1254-1255* locus. Expression of this locus is 2.1-fold induced in the wildtype under aerobic growth conditions and 3.7-fold in the NFR-deletion derivative. A single deletion of this NOX does not impact H₂O₂ production of the wildtype, but completely abolishes all H₂O₂ production in the NFR deletion derivative, also after prolonged oxygen exposure. Surprisingly, this mutant also shows hampered growth and lower biomass yield in the presence of oxygen, despite its H₂O₂-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 extensive growth stagnation observed during growth under a N₂ atmosphere, observed both in liquid (sparged batch cultures) as on solid media (Anopore™ slides). The cause of this growth stagnation is a lack of CO₂ which *L. johnsonii* requires for growth. Two aspects of this observation are unexpected. Firstly, oxygen can fully relieve this CO₂ growth dependency. Secondly, lack of CO₂ apparently leads to cell death of *L. johnsonii*, which 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 show that this oxygen relieve of CO₂ dependency also counts for the acetate growth dependency: *L. johnsonii* cannot grow in an environment without acetate unless the culture is aerated. Both these effects of oxygen can 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 aerobically. A pyruvate oxidase deletion derivative confirms this hypothesis as it renders 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 make an attempt to further understand the metabolic requirement for CO₂ by analyzing genome-wide transcriptome changes in an anaerobically growing culture upon CO₂ depletion. We observe an extensive rearrangement of gene expression, including many transporters and regulators. Additionally, expression of the *pyr*-encoded pyrimidine biosynthesis pathway, also referred to as the carbamoyl-phosphate pathway, is strongly upregulated (up to 17-fold). In other LAB, this pathway was associated with CO₂ dependency, which is required in the first enzymatic step. Growth stagnation due to CO₂ removal could be prevented by addition of sufficient levels of pyrimidines. However, in *L. johnsonii* such a relation between pyrimidine

supplementation and CO₂ dependency is not observed. Supplementation of other compounds previously associated with CO₂ growth dependency in LAB, such as arginine and aspartate could neither prevent CO₂ induced growth stagnation. We speculate that *L. johnsonii* may be unable to incorporate exogenous pyrimidines and propose further experiments to test this proposition.

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 remain 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 economically 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 scavenged by neighboring catalase-expressing bacteria. We propose several experiments to study this hypothesis.

Lastly, we discuss how hydrogen peroxide production and aerotolerance 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, H₂O₂-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 H₂O₂ in host/microbiome interactions.