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Impact of Respiration on Resistance of Lactobacillus plantarum WCFS1 to Acid Stress

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This study shows that growth under respiration conditions has a negative impact on the survival of stationary-phase cultures of Lactobacillus plantarum WCFS1 at low pHs and that viability loss at critical values is associated with the formation of radicals and loss of membrane integrity.

Lactobacillus plantarum is a facultative anaerobic bacterium that is used as a model organism in studies on microbe-host interactions for identification of probiotic functions. L. plantarum WCFS1, which was isolated from human saliva, is one of the most widely studied strains, and its genome has been sequenced. The function of this strain in the gastrointestinal tract has been investigated extensively, and specific host immune responses have been described. Probiotics encounter various adverse conditions during processing, storage, and passage through the gastrointestinal tract, and it is therefore essential to increase their robustness for improved functionality. Recently, it was shown that L. plantarum is capable of respiration under aerobic conditions in the presence of heme and menaquinone, which resulted in an increase in biomass formation. Furthermore, we previously showed that respiration provided resistance to hydrogen peroxide. In this study, we investigated the acid resistance of L. plantarum WCFS1 grown in MRS broth under fermentation, aerobic, aerobic with hemin, and respiration conditions. Furthermore, we assessed the relationship between acid resistance, radical formation, and cytoplasmic membrane permeability of L. plantarum WCFS1.

L. plantarum WCFS1 was cultivated in MRS broth (Merck, Germany). Growth under respiration conditions was induced by addition of hemin (Sigma-Aldrich) and vitamin K2 (Sigma-Aldrich) to final concentrations of 10 μg ml−1 and 50 μg ml−1, respectively, allowing induction of electron transfer chain (ETC) activity. To prepare precultures, 10 ml of MRS was statically incubated overnight at 30°C. Precultures were washed once with phosphate-buffered saline (PBS; pH adjusted to 7.4 with HCl), resuspended in a similar volume of PBS, and inoculated into the different media (1% [vol/vol]). Cultures were grown under fermentation (microaerobic or static) or aerobic (shaking) conditions at 30°C. Aerobic growth was achieved by growing in a shake flask (100 ml) at 200 rpm. Stress survival, radical formation, and membrane integrity of stationary-phase cultures (25 h) were assessed after exposure to low pH. The pH of the cultures was adjusted using 18% hydrochloric acid, and the cultures were subsequently incubated at 30°C for 30 min. After acid exposure, samples were serially diluted in peptone physiological salt solution (PPS; 1 g of neutralized bacteriological peptone liter−1 [Oxoid, England] and 8.5 g of NaCl liter−1 in water), and appropriate dilutions were plated on MRS agar plates (Merck, Darmstadt, Germany). Plates were incubated for 72 h at 30°C, and colonies were enumerated. All stress survival experiments were performed with three independent biological replicates. The mean values and standard deviations were calculated, and statistically significant differences were determined using the Student t test. Radical formation was measured as described previously using dihydroethidium (DHE; Invitrogen, Leiden, The Netherlands). Membrane integrity was assessed by using propidium iodide (PI; Invitrogen, Leiden, The Netherlands) following the manufacturer’s protocol. Stained samples were run on a Becton Dickinson FACSCalibur flow cytometer with the following photomultiplier tube (PMT) voltage settings: E00 (forward scatter [FSC]), 350 (side scatter [SSC]), 650 (fluorescence channel 2 [FL2]) and 650 (fluorescence channel 3 [FL3]). Data were obtained from 20,000 cells at a medium flow rate using Cellquest Pro (version 4.0.2) and

![Graph showing acid resistance of L. plantarum WCFS1 grown in MRS broth under fermentation, aerobic, aerobic with hemin, and respiration conditions.](https://aem.asm.org/article-pdf/78/11/4062/3303082/4062.pdf)
subsequently analyzed with WinMDI 2.9 (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA; http://facs.scripps.edu/software.html).

To investigate the effect of oxygen utilization on the resistance to acid stress, stationary-phase cells of cultures grown in MRS were exposed to low pH stress (Fig. 1). Respiring cells were most sensitive to acid exposure, while fermentative cells were most acid resistant. Interestingly, for all growth modes, a decrease of only 0.15 pH unit resulted in drastic reduction in acid survival. This critical pH was lowest for fermenting cells (pH 2.0) and highest for respiring cells (pH 2.30), while aerobic growth conditions with and without hemin yielded a critical pH of pH 2.15. Previously, studies using fluorescent dyes showed that acid exposure of Bacillus cereus could result in intracellular radical formation and cell death (8, 9). Therefore, we performed flow cytometry experiments at decreasing pHs to assess radical formation and cell membrane integrity, using the dyes dihydroethidium (Fig. 2) and propidium iodide (Fig. 3), respectively. Exposure to decreasing pHs resulted in an increase in radical formation and a decrease in membrane integrity. The increase of radical formation and decrease in membrane integrity were gradual for fermenting cells, while for aerobic and respiring cells a more pronounced increase in radical formation and decrease in membrane integrity were observed at the critical pH, indicating that acid resistance is related to these factors. Furthermore, the differences in acid survival of the different growth modes at decreasing pHs roughly corresponded with the level of radical formation and membrane integrity, with the respiring cells showing a shift at the highest pH. Apparently, the respiratory condition is most prone to radical formation, which is most likely the result of the disturbance of the ETC and subsequent leakage of electrons from intermediates of the ETC (7). Radicals can cause significant damage to various cellular structures, such as DNA, proteins, and phospholipids (4). Our data are in contrast with data on acid survival of Lactococcus lactis grown under respiration conditions (10). It is conceivable that differences in production of superoxide dismutase and (heme) catalase contribute to these responses (1, 10, 15).

Our results suggest that sudden death at critical low pHs is associated with the formation of radicals and loss of membrane integrity. The information obtained may provide tools for optimization of acid survival by modulation of growth conditions and strain selection.

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10. Rezaie L, et al. 2004. Respiration metabolism reduces oxidative and acid stress and radical formation during acid exposure of L. plantarum WCFS1. The graphs present the distribution of the fluorescent signal of 20,000 stationary-phase cells (events) of L. plantarum WCFS1 grown under fermentative (F), aerobic (S), aerobic with hemin (S+H), and respiratory (R) conditions at 30°C before stress (dark blue) and after 30 min exposure to pH 2.45 (light blue), 2.30 (green), 2.15 (yellow), and 2.00 (red). The formation of radicals is indicated with a shift to the right (increase) of the fluorescent signal.

FIG 2 Effect of growth conditions on radical formation during acid exposure of L. plantarum WCFS1. The graphs present the distribution of the fluorescent signal of 20,000 stationary-phase cells (events) of L. plantarum WCFS1 grown under fermentative (F), aerobic (S), aerobic with hemin (S+H), and respiratory (R) conditions at 30°C before stress (dark blue) and after 30 min exposure to pH 2.45 (light blue), 2.30 (green), 2.15 (yellow), and 2.00 (red). The formation of radicals is indicated with a shift to the right (increase) of the fluorescent signal.

