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Seshagiri Rao, Nikhil

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NIKHIL SESHAGIRI RAO
APPLIED MICROBIOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY

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Impact of Cultivation Parameters on Cell Physiology of *Limosilactobacillus reuteri*

Nikhil Seshagiri Rao

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Department of Food Science, University of Copenhagen,

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Abstract: Optimisation of cultivation conditions in the industrial production of probiotics is crucial to reach a high-quality product with high probiotic functionality. The production process includes a fermentation step to produce biomass, accompanied by centrifugation to concentrate the cells. Subsequently, the cells are treated with stabilising solutions (lyoprotectants) before they are subjected to freezing. The frozen cell pellets can be subjected to freeze-drying to yield a dried final product. The probiotic product needs to withstand adverse environmental conditions both during production and after consumption (gastro-intestinal tract).

The objective of this study was to elucidate the cellular response to various production process parameters and evaluate their influence on freeze-drying tolerance. In addition, the stability and probiotic activity of freeze-drying product was studied. Parameters such as temperature, pH, oxygen, media components during fermentation, and the pre-formulation hold time prior to freeze-drying were in focus.

Furthermore, flow cytometry-based descriptors of bacterial morphology were evaluated for their potential correlation with process-relevant output parameters and physiological fitness during cultivation to avoid suboptimal growth. Additionally, a pipeline was developed for online flow cytometry combined with automated data processing using the k-means clustering algorithm, which is a promising process analytical technology tool. The effects of temperature, initial pH, and oxygen levels on cell growth and cell size distributions of *Limosilactobacillus reuteri* DSM 17938 were investigated using multivariate flow cytometry. Morphological heterogeneities were observed under non-optimal growth conditions, with low temperature, high initial pH, and high oxygen levels triggering changes in morphology towards cell chain formation. High-growth pattern characterised by smaller cell sizes and decreased population heterogeneity was observed using the pulse width distribution parameter. This parameter can be used to distinguish larger cells from smaller cells and to separate singlets from doublets (i.e., single cells from aggregated cells). Although, oxygen is known to inhibit growth in *L. reuteri*, controlled oxygen supply resulted in noticeable effect on the cell metabolism, in a higher degree of unsaturated fatty acids in the cell, and improved freeze-drying stress tolerance. Another important component that was examined was the addition of exogeneous fatty acid source in the form of Tween 80. A chemically defined minimal medium was developed, with 14 amino acids identified as essential for growth. The addition of Tween 80 to the medium improved biomass yield, growth rate, and shortened cultivation time. *L. reuteri* DSM 17938 may not be able to efficiently synthesise unsaturated fatty acid without an exogenous fatty acid source, but this requires further investigation. Lastly, the pre-formulation hold time during the manufacture of probiotics was found to significantly affect long-term stability, with direct freeze samples showing better freeze-drying stability compared to those subjected to rest for 3 h incubation at room temperature. These findings suggest that an optimised production process and formulation of agents can lead to the successful production of high-quality probiotics with excellent stability.

Key words: Probiotics, *L. reuteri*, oxygen, fermentation, heterogeneity, morphology, flow cytometry, freeze-drying stress tolerance, online flow cytometry, k-mean clustering, fatty acids, defined minimal media, pre-formulation hold time.

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LUND
UNIVERSITY

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Division of Applied Microbiology

Department of Chemistry

Faculty of Engineering

Lund University

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Table of Contents

Abstract

Optimisation of cultivation conditions in the industrial production of probiotics is crucial to reach a high-quality product with high probiotic functionality. The production process includes a fermentation step to produce biomass, accompanied by centrifugation to concentrate the cells. Subsequently, the cells are treated with stabilising solutions (lyoprotectants) before they are subjected to freezing. The frozen cell pellets can be subjected to freeze-drying to yield a dried final product. The probiotic product needs to withstand adverse environmental conditions both during production and after consumption (gastro-intestinal tract).

The objective of this study was to elucidate the cellular response to various production process parameters and evaluate their influence on freeze-drying tolerance. In addition, the stability and probiotic activity of freeze-drying product was studied. Parameters such as temperature, pH, oxygen, media components during fermentation, and the pre-formulation hold time prior to freeze-drying were in focus.

Furthermore, flow cytometry-based descriptors of bacterial morphology were evaluated for their potential correlation with process-relevant output parameters and physiological fitness during cultivation to avoid suboptimal growth. Additionally, a pipeline was developed for online flow cytometry combined with automated data processing using the k-means clustering algorithm, which is a promising process analytical technology tool. The effects of temperature, initial pH, and oxygen levels on cell growth and cell size distributions of *Limosilactobacillus reuteri* DSM 17938 were investigated using multivariate flow cytometry. Morphological heterogeneities were observed under non-optimal growth conditions, with low temperature, high initial pH, and high oxygen levels triggering changes in morphology towards cell chain formation. High-growth pattern characterised by smaller cell sizes and decreased population heterogeneity was observed using the pulse width distribution parameter. This parameter can be used to distinguish larger cells from smaller cells and to separate singlets from doublets (i.e., single cells from aggregated cells). Although, oxygen is known to inhibit growth in *L. reuteri*, controlled oxygen supply resulted in noticeable effect on the cell metabolism, in a higher degree of unsaturated fatty acids in the cell, and improved freeze-drying stress tolerance. Another important component that was examined was the addition of exogeneous fatty acid source in the form of Tween 80. A chemically defined minimal medium was developed, with 14 amino acids identified as essential for growth. The addition of

Tween 80 to the medium improved biomass yield, growth rate, and shortened cultivation time. *L. reuteri* DSM 17938 may not be able to efficiently synthesise unsaturated fatty acid without an exogenous fatty acid source, but this requires further investigation. Lastly, the pre-formulation hold time during the manufacture of probiotics was found to significantly affect long-term stability, with direct freeze samples showing better freeze-drying stability compared to those subjected to rest for 3 h incubation at room temperature. These findings suggest that an optimised production process and formulation of agents can lead to the successful production of high-quality probiotics with excellent stability.

Popular scientific summary

Did you know that there are more bacterial cells than human cells in the human body? Most bacteria are found in a person's gastro-intestinal tract, which includes the mouth, oesophagus, stomach, small intestine, and large intestine. These bacterial communities determine how you feel in general. If the bacterial community is disturbed, for example by an antibiotic treatment, one can run the risk of being attacked by pathogens, which are the unwanted type of bacteria. To maintain the balance between microbes in the gastro-intestinal tract, probiotics can be used as supplements. Probiotics are live microorganisms that, when administered in sufficient amounts, provide a health benefit.

I have been studying a probiotic bacterium called *Limosilactobacillus reuteri* DSM 17938, which has been shown to have benefits in fighting many gastro-intestinal ailments, improving immunity and oral health. The aim of my thesis work was to understand how *L. reuteri* reacts to process parameters during the cultivation of bacteria that have a significant impact on the stability of the probiotic product. For a probiotic product to be fully effective, it must survive the stresses it goes through during manufacturing, be stable during storage and when it is exposed to stress during its passage through the human body, e.g., resist acid stress, bile stress, while producing compounds that improve immunity.

The probiotic bacteria were grown under different culture conditions to create a variation in growth and morphology. The bacteria were monitored using an instrument called a flow cytometer. I developed new flow cytometer-based ways to study the bacteria, which were grown in fermenters. An online instrument combined with automated data analysis was developed to better analyse the culture at the single-cell level that could measure cell number, viability (% viable cell population), and cell size and shape (morphology) all at the same time. New morphology descriptor, pulse width was used to separate smaller cells from longer cells. The utilization of pulse width to describe morphological heterogeneity in fermentation processes has not been previously documented. The cells were converted to powder form by freeze-drying, a process where all water in the sample is removed under the application of vacuum, making it more stable for storage. The bacteria were counted before and after the freeze-drying process and provided information on the survival of the bacteria. Survival was correlated with different culture conditions in which the bacteria were grown to understand the best conditions for probiotic production. In conclusion, by creating a variation, I was able to screen many conditions and their impact on the final probiotic freeze-dried product. To my knowledge, temperature

and oxygen had the greatest impact on the bacteria. There were alterations in growth, products formed during cultivation and lipid composition of the bacteria. The results indicate that refining the production procedure can result in the effective production of quality probiotics that demonstrate remarkable stability.

Populärvetenskaplig sammanfattning

Visste du att det finns fler bakterieceller än mänskliga celler i människokroppen? De flesta bakterierna finns i en människas mag-tarmkanal, som omfattar mun, matstrupe, magsäck, tunntarm och tjocktarm. Dessa bakteriesamhällen bestämmer hur du känner dig i allmänhet. Om bakteriesamhället störs, till exempel av en antibiotikabehandling, kan man löpa risk för angrepp av patogener, som är den oönskade typen av bakterier. För att hålla balansen mellan mikrober i mag-tarm kanalen kan man använd probiotika som tillskott. Probiotika är levande mikroorganismer som, när de administreras i tillräckliga mängder, ger en hälsofördel.

Jag har studerat en probiotisk bakterie vid namn *Limosilactobacillus reuteri* DSM 17938, som har visat sig ha fördelar mot att bekämpa många mag-tarmkanalrelaterade åkommor, förbättra immunitet och munhälsa. Syftet med mitt avhandlingsarbete var att förstå hur *L. reuteri* reagerar på processparametrar under odling av bakterier som hat en betydande inverkan på stabiliteten hos den probiotiska produkten. För att en probiotisk produkt ska vara fullt effektiv måste den överleva de påfrestningar som den går genom under tillverkningen vara stabil under lagring och då den utsätts för stress under sin passage genom människokroppen, tex. motstå sur stress, gallstress, samtidigt som den producerar föreningar som förbättrar immuniteten.

De probiotiska bakterierna odlades under olika odlingsförhållanden för att skapa en variation i tillväxt och morfologi. Bakterierna övervakades med ett instrument som kallas flödescytometer. Jag utvecklade nya flödescytometerbaserade sätt att studera bakterierna, som odlades i fermentorer. Ett onlineinstrument kombinerat med automatiserad dataanalys utvecklades för att bättre analysera kulturen på encellsnivå som kunde mäta cellantal, livsduglighet (% levande cellpopulation) och cellstorlek och form (morfologi) allt på samma gång. En ny morfologideskriptor, pulsbredd, användes för att separera mindre celler från längre celler. Användningen av pulsbredd för att beskriva morfologisk heterogenitet i fermenteringsprocesser har inte dokumenterats tidigare. Cellerna omvandlades till pulverform genom frystorkning, en process där allt vatten i provet avlägsnas under applicering av vakuum, vilket gör det mer stabilt för lagring. Bakterierna räknades före och efter frystorkningsprocessen och gav information om bakteriernas överlevnad. Överlevnad korrelerades med olika odlingsförhållanden som bakterierna odlades på för att förstå de bästa förutsättningarna för probiotisk produktion. Sammanfattningsvis, genom att skapa en variation, kunde jag screena många

tillstånd och deras inverkan på den slutliga probiotiska frystorkningsprodukten. Såvitt jag vet hade temperatur och syre störst påverkan på bakterierna. Det förekom förändringar i tillväxten, produkter som bildades under odlingen och lipidsammansättningen av bakterierna. Resultaten indikerar att förfining av produktionsproceduren kan resultera i effektiv produktion av kvalitetsprobiotika som uppvisar anmärkningsvärd stabilitet.

ಜನಪ್ರಿಯ ವೈಜ್ಞಾನಿಕ ಸಾರಾಂಶ (Kannada)

ಮಾನವ ದೇಹದಲ್ಲಿ ಮಾನವ ಜೀವಕೋಶಗಳಿಗಿಂತ ಹೆಚ್ಚು ಬ್ಯಾಕ್ಟೀರಿಯಾದ ಜೀವಕೋಶಗಳಿವೆ ಎಂದು ನಿಮಗೆ ತಿಳಿದಿದೆಯೇ? ಹೆಚ್ಚಿನ ಬ್ಯಾಕ್ಟೀರಿಯಾಗಳು ಬಾಯಿ, ಅನ್ನನಾಳ, ಹೊಟ್ಟೆ, ಸಣ್ಣ ಕರುಳು ಮತ್ತು ದೊಡ್ಡ ಕರುಳನ್ನು ಒಳಗೊಂಡಿರುವ ಮಾನವನ ಜಠರಗರುಳಿನ ಪ್ರದೇಶದಲ್ಲಿವೆ. ಈ ಬ್ಯಾಕ್ಟೀರಿಯಾದ ಸಮುದಾಯಗಳು ನೀವು ಸಾಮಾನ್ಯವಾಗಿ ಹೇಗೆ ಭಾವಿಸುತ್ತೀರಿ ಎಂಬುದನ್ನು ನಿರ್ದೇಶಿಸಬಹುದು. ಬ್ಯಾಕ್ಟೀರಿಯಾದ ಸಮುದಾಯವು ಅಡ್ಡಿಪಡಿಸಿದರೆ ಅಥವಾ ತೊಂದರೆಗೊಳಗಾದರೆ, ಉದಾಹರಣೆಗೆ, ಪ್ರತಿಜೀವಕ ಚಿಕಿತ್ಸೆಗಳಿಂದ, ಅನಪೇಕ್ಷಿತ ರೀತಿಯ ಬ್ಯಾಕ್ಟೀರಿಯಾಗಳಾದ ರೋಗಕಾರಕಗಳಿಂದ ಆಕ್ರಮಣಕ್ಕೆ ಒಳಗಾಗುವ ಅಪಾಯವಿದೆ. ಕರುಳಿನ ಸೂಕ್ಷ್ಮಜೀವಿಗಳ ಸಮತೋಲನವನ್ನು ಕಾಪಾಡಿಕೊಳ್ಳಲು, ಪ್ರೋಬಯಾಟಿಕ್ಗಳನ್ನು ಪೂರಕಗಳಾಗಿ ಬಳಸಲಾಗುತ್ತದೆ. ಪ್ರೋಬಯಾಟಿಕ್ಗಳು ಲೈವ್ ಸೂಕ್ಷ್ಮಜೀವಿಗಳಾಗಿದ್ದು, ಸಾಕಷ್ಟು ಪ್ರಮಾಣದಲ್ಲಿ ನಿರ್ವಹಿಸಿದಾಗ, ಹೋಸ್ಟ್ಗೆ ಆರೋಗ್ಯ ಪ್ರಯೋಜನವನ್ನು ನೀಡುತ್ತದೆ.

ಈ ಪ್ರಬಂಧದಲ್ಲಿ, *ಲಿಮೋಸಿಲಾಕ್ಟೊಬ್ಯಾಸಿಲಸ್ ರಿಯುಟೆರಿ* ಡಿಎಸ್ಎಮ್ 17938 ಎಂಬ ಹೆಸರಿನ ಪ್ರೋಬಯಾಟಿಕ್ ಬ್ಯಾಕ್ಟೀರಿಯಂ ಅನ್ನು ಚೆನ್ನಾಗಿ ಅಧ್ಯಯನ ಮಾಡಲಾಗಿದೆ, ಇದು ಜಠರಗರುಳಿನ ಪ್ರದೇಶಕ್ಕೆ ಸಂಬಂಧಿಸಿದ ಅನೇಕ ಕಾಯಿಲೆಗಳ ವಿರುದ್ಧ ಹೋರಾಡುವ ಪ್ರಯೋಜನಗಳನ್ನು ಹೊಂದಿದೆ ಎಂದು ತೋರಿಸಲಾಗಿದೆ, ರೋಗನಿರೋಧಕ ಶಕ್ತಿಯನ್ನು ಸುಧಾರಿಸುತ್ತದೆ ಮತ್ತು ಬಾಯಿಯ ಆರೋಗ್ಯವನ್ನು ಸುಧಾರಿಸುತ್ತದೆ. ಪ್ರೋಬಯಾಟಿಕ್ ಉತ್ಪನ್ನದ ಸ್ಥಿರತೆಯ ಮೇಲೆ ಗಮನಾರ್ಹ ಪರಿಣಾಮ ಬೀರುವ ಉತ್ಪಾದನೆಯ ಸಮಯದಲ್ಲಿ ಪ್ರಕ್ರಿಯೆಯ ನಿಯತಾಂಕಗಳಿಗೆ ಕೋಶವು ಹೇಗೆ ಪ್ರತಿಕ್ರಿಯಿಸುತ್ತದೆ ಎಂಬುದನ್ನು ಅರ್ಥಮಾಡಿಕೊಳ್ಳುವುದು ಯೋಜನೆಯ ಗುರಿಯಾಗಿದೆ. ಪ್ರೋಬಯಾಟಿಕ್ ಉತ್ಪನ್ನವು ಸಂಪೂರ್ಣವಾಗಿ ಪರಿಣಾಮಕಾರಿಯಾಗಿರಲು, ಅದು ಉತ್ಪಾದನಾ ಪೈಪ್ಲೈನ್ ಮೂಲಕ ಹಾದುಹೋಗುವ ಒತ್ತಡಗಳನ್ನು ಬದುಕಬೇಕು, ಶೇಖರಣೆಯ ಸಮಯದಲ್ಲಿ ಸ್ಥಿರವಾಗಿರಬೇಕು ಮತ್ತು ಆಮ್ಲ ಒತ್ತಡ, ಪಿತ್ತರಸದ ಒತ್ತಡವನ್ನು ತಡೆದುಕೊಳ್ಳುವ ಮೂಲಕ ಮಾನವ ದೇಹದ ಮೂಲಕ ಹಾದುಹೋಗಬೇಕು, ಅದರ ನೇರ ಸ್ಥಿತಿಯನ್ನು ಉಳಿಸಿಕೊಂಡು ಸಂಯುಕ್ತಗಳನ್ನು ಉತ್ಪಾದಿಸಬೇಕು. ಅದು ರೋಗನಿರೋಧಕ ಶಕ್ತಿಯನ್ನು ಸುಧಾರಿಸುತ್ತದೆ.

ಪ್ರೋಬಯಾಟಿಕ್ ಬ್ಯಾಕ್ಟೀರಿಯಾವನ್ನು ವಿವಿಧ ಸಂಸ್ಕೃತಿಯ ಪರಿಸ್ಥಿತಿಗಳಲ್ಲಿ (ತಾಪಮಾನ, ಪಿಹೆಚ್, ಆಮ್ಲಜನಕ, ಇತ್ಯಾದಿ) ಬೆಳವಣಿಗೆ ಮತ್ತು ರೂಪವಿಜ್ಞಾನದಲ್ಲಿ ವ್ಯತ್ಯಾಸವನ್ನು ಸೃಷ್ಟಿಸಲು ಬೆಳೆಸಲಾಗುತ್ತದೆ. ಫ್ಲೋ ಸೈಟೋಮೀಟರ್ ಎಂಬ ಉಪಕರಣವನ್ನು ಬಳಸಿಕೊಂಡು ಅಸ್ಥಿರಗಳನ್ನು ಮೇಲ್ವಿಚಾರಣೆ ಮಾಡಲಾಯಿತು. ನಾನು ಬ್ಯಾಕ್ಟೀರಿಯಾವನ್ನು ಅಧ್ಯಯನ ಮಾಡಲು ಹೊಸ ಫ್ಲೋ ಸೈಟೋಮೀಟರ್-ಆಧಾರಿತ

ವಿಧಾನಗಳನ್ನು ಅಭಿವೃದ್ಧಿಪಡಿಸಿದೆ, ಇದನ್ನು ಹುದುಗುವಿಕೆಗಳಲ್ಲಿ ಬೆಳೆಸಲಾಗುತ್ತದೆ. ಸೆಲ್ ಸಂಖ್ಯೆ, ಕಾರ್ಯಸಾಧ್ಯತೆ (% ಲೈವ್ ಸೆಲ್ ಜನಸಂಖ್ಯೆ), ಮತ್ತು ಜೀವಕೋಶದ ಗಾತ್ರ ಮತ್ತು ಆಕಾರ (ರೂಪವಿಜ್ಞಾನ) ಎಲ್ಲವನ್ನೂ ಒಂದೇ ಸಮಯದಲ್ಲಿ ಅಳೆಯುವ ಏಕ-ಕೋಶ ಮಟ್ಟದಲ್ಲಿ ಸಂಸ್ಕೃತಿಯನ್ನು ಉತ್ತಮವಾಗಿ ವಿಶ್ಲೇಷಿಸಲು ಸ್ವಯಂಚಾಲಿತ ಡೇಟಾ ವಿಶ್ಲೇಷಣೆಯೊಂದಿಗೆ ಸಂಯೋಜಿಸಲಾದ ಆನ್‌ಲೈನ್ ಉಪಕರಣವನ್ನು ಅಭಿವೃದ್ಧಿಪಡಿಸಲಾಗಿದೆ. ಕೋಶಗಳನ್ನು ಫ್ರೀಜ್-ಒಣಗಿಸುವ ಮೂಲಕ ಪುಡಿ ರೂಪಕ್ಕೆ ಪರಿವರ್ತಿಸಲಾಯಿತು, ಈ ಪ್ರಕ್ರಿಯೆಯಲ್ಲಿ ಮಾದರಿಯಲ್ಲಿರುವ ಎಲ್ಲಾ ನೀರನ್ನು ನಿರ್ವಾತದ ಅನ್ವಯದ ಅಡಿಯಲ್ಲಿ ತೆಗೆದುಹಾಕಲಾಗುತ್ತದೆ, ಇದು ಶೇಖರಣೆಗಾಗಿ ಹೆಚ್ಚು ಸ್ಥಿರವಾಗಿರುತ್ತದೆ. ಫ್ರೀಜ್-ಒಣಗಿಸುವ ಪ್ರಕ್ರಿಯೆಯ ಮೊದಲು ಮತ್ತು ನಂತರ ಬ್ಯಾಕ್ಟೀರಿಯಾವನ್ನು ಎಣಿಸಲಾಯಿತು ಮತ್ತು ಬ್ಯಾಕ್ಟೀರಿಯಾದ ಬದುಕುಳಿಯುವಿಕೆಯ ಬಗ್ಗೆ ಮಾಹಿತಿಯನ್ನು ಒದಗಿಸಲಾಯಿತು. ಬದುಕುಳಿಯುವಿಕೆಯು ವಿಭಿನ್ನ ಸಂಸ್ಕೃತಿಯ ಪರಿಸ್ಥಿತಿಗಳೊಂದಿಗೆ ಪರಸ್ಪರ ಸಂಬಂಧ ಹೊಂದಿದೆ, ಪ್ರೋಬಯಾಟಿಕ್ ಉತ್ಪಾದನೆಗೆ ಉತ್ತಮ ಪರಿಸ್ಥಿತಿಗಳನ್ನು ಅರ್ಥಮಾಡಿಕೊಳ್ಳಲು ಬ್ಯಾಕ್ಟೀರಿಯಾವನ್ನು ಬೆಳೆಸಲಾಯಿತು. ಕೊನೆಯಲ್ಲಿ, ಬದಲಾವಣೆಯನ್ನು ರಚಿಸುವ ಮೂಲಕ, ನಾನು ಅನೇಕ ಪರಿಸ್ಥಿತಿಗಳನ್ನು ಮತ್ತು ಅಂತಿಮ ಪ್ರೋಬಯಾಟಿಕ್ ಫ್ರೀಜ್-ಒಣಗಿಸುವ ಉತ್ಪನ್ನದ ಮೇಲೆ ಅವುಗಳ ಪ್ರಭಾವವನ್ನು ಪ್ರದರ್ಶಿಸಲು ಸಾಧ್ಯವಾಯಿತು. ನನ್ನ ಜ್ಞಾನಕ್ಕೆ, ಆಮ್ಲಜನಕವು ಬ್ಯಾಕ್ಟೀರಿಯಾದ ಮೇಲೆ ಹೆಚ್ಚಿನ ಪರಿಣಾಮವನ್ನು ಬೀರುತ್ತದೆ ಮತ್ತು ಅದರ ಪ್ರಯೋಜನವನ್ನು ಅರ್ಥಮಾಡಿಕೊಳ್ಳಲು ಹೆಚ್ಚಿನ ಅಧ್ಯಯನಗಳನ್ನು ಕೈಗೊಳ್ಳಬೇಕಾಗಿದೆ.

List of Papers

This thesis is based on the following publications and manuscripts, which will be referred to using Roman numerals:

I. **Flow cytometric analysis reveals culture condition dependent variations in phenotypic heterogeneity of *Limosilactobacillus reuteri***

Nikhil Seshagiri Rao, Ludwig Lundberg, Shuai Palmkron, Sebastian Håkansson, Björn Bergenståhl, Magnus Carlquist. (2021), In: *Scientific Reports*, 11, 23567. https://doi.org/10.1038/s41598-021-02919-3

II. **Non-inhibitory levels of oxygen during cultivation increase freeze-drying stress tolerance in *Limosilactobacillus reuteri* DSM 17938**

Nikhil Seshagiri Rao, Ludwig Ermann Lundberg, Julia Tomasson, Cecilia Tullberg, Daniel P. Brink, Shuai Bai Palmkron, Ed WJ van Niel, Sebastian Håkansson, Magnus Carlquist. (2023), *Accepted. Frontiers in Microbiology*. doi: 10.3389/fmicb.2023.1152389

III. **Impact of non-fat growth medium on the lipid composition in *Limosilactobacillus reuteri* DSM 17938**

Nikhil Seshagiri Rao, Cecilia Tullberg, Sebastian Håkansson, Christer Larsson, Ed WJ van Niel, Magnus Carlquist. *Manuscript*

IV. **Impact of pre-formulation hold time on long-term stability of *Limosilactobacillus reuteri* DSM 17938**

Nikhil Seshagiri Rao, Shuai Bai Palmkron, Ludwig Ermann Lundberg, Krishnan Sreenivas, Christer Larsson, Björn Bergenståhl, Anna Millqvist Fureby, Ed WJ van Niel, Stefan Roos, Magnus Carlquist, Sebastian Håkansson. *Manuscript*

My contribution to the Papers

I. I participated in the study design along with my supervisor. I performed the experimental work together with a master's student. I performed flow cytometric analysis and, data analysis as well as drafted the manuscript.

II. I participated in the study design along with my supervisor. I carried out all the batch cultivations in the bioreactor. I set up the online flow cytometry. I performed analytical techniques such as HPLC and FAME analysis. I performed the data analysis and drafted the manuscript.

III. I participated in the study design along with my supervisor. I developed a defined media. I carried out all the batch cultivations in the bioreactor and performed analytical techniques such as HPLC and FAME analysis. I completed the data analysis and drafted the manuscript.

IV. I participated in the study design along with all the authors. I carried out all the batch cultivations in the bioreactor together with another co-author. I performed flow cytometry, HPLC, and shelf-life study analysis. I performed the data analysis together with the other co-authors and drafted the manuscript.

Abbreviations

LAB – Lactic acid bacteria

MRS – De Man, Rogosa and Sharpe

FCM – Flow cytometry

FSC – Forward scatter

SSC – Side scatter

FL – Fluorescence

PI – Propidium Iodide

SG – SYBR Green

CFDA-SE – Carboxyfluorescein diacetate succinimidyl ester

FD – Freeze-drying

GIT – Gastro-intestinal tract

CO2 – Carbon dioxide

FA – Fatty acid

ACP – Acyl carrier protein

UFA – Unsaturated fatty acid

SFA – Saturated fatty acid

CFA – Cyclic fatty acid

PKP – Phosphoketolase pathway

EMP – Embden-Meyerhof-Parnas pathway

NAD(P)H – Nicotinamide adenine dinucleotide (phosphate), reduced

NAD(P)+ – Nicotinamide adenine dinucleotide (phosphate), oxidised

CoA – Coenzyme A

ATP – Adenosine triphosphate

ADP – Adenosine diphosphate

ROS – Reactive oxygen species

CFU – Colony forming unit

AFU – Active fluorescence unit

PAT – Process analytical technology

HPLC – High performance liquid chromatography

GC – Gas chromatography

CV – Coefficient of variation

rCV – Robust coefficient of variation

1. Introduction

Gut microbiota

Gut microbiota, also known as gut flora, are an incredibly complex and diverse community of microorganisms ranging from actinobacteria, firmicutes, fusobacteria, proteobacteria, and other phyla that live in the gastro-intestinal tract (GIT) of all humans and many other animals (Milani et al., 2017; Rodríguez et al., 2015). Microbiota benefit the host in a variety of ways, such as by promoting gut health (Natividad & Verdu, 2013), supplying energy (Den Besten et al., 2013), warding off pathogens (Baümler & Sperandio, 2016), and controlling immunity (Gensollen et al., 2016). Disruption to the microbial composition, known as dysbiosis, can, however, lead to these beneficial functions being negatively impacted.

The number of publications regarding gut microbiota in health and diseases are on the rise, highlighting interest in the research topic.[1] Colonisation of the gut starts immediately from the time of birth. Upon passing through the birth canal, new-borns encounter a diverse range of microorganisms from actinobacteria, firmicutes, fusobacteria, proteobacteria and other phyla. The composition of the microorganisms in the GIT can be altered rapidly due to circumstances such as illness, antibiotic treatment, and modifications to one's diet and lifestyle (David et al., 2014; Donaldson et al., 2015; Huang et al., 2015; Koenig et al., 2011; Rodríguez et al., 2015; Walker et al., 2011). Administering live bacteria to positively alter the microbiota-health relationship has sparked interest as a potential means of preventing or even curing certain diseases.

Probiotics

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit to the host (Hill et al., 2014). Probiotic bacteria are the same

[1] Number of publications related to the intestinal microbiota in 2008 was 1161, whereas in 2022 it rose to 57427. Data were obtained by searching PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) with the following terms: intestinal microbiota, gut microbiota, intestinal flora, gut microflora.

as beneficial microorganisms found in the human gut. They are often referred to as 'good' or 'helpful' bacteria because they are thought to help restore the balance of bacteria in the digestive system, which can be beneficial for digestion, immunity, and overall health. Table 1 depicts the health benefits of ingesting probiotic bacteria.

Table 1. The evidence in support of the health benefits of taking probiotics.

Benefit	Probiotic bacteria	References
Lowered irritable bowel syndrome	*Saccharomyces cerevisiae* CNCM I-3856, *Bifidobacterium infantis*,	(Cayzeele-Decherf et al., 2017; Yuan et al., 2017; Zhang et al., 2016)
Inflammatory bowel diseases, such as pouchitis and Crohn's disease	Probitic cocktail, *Lactobacillus* GG	(Gionchetti et al., 2000; Gupta et al., 2000)
Reduction in infant colic	*Lactobacillus reuteri* DSM 17938	(Chau et al., 2015; Liu et al., 2019; Roos et al., 2013; Srinivasan et al., 2018)
Prevention of necrotising enterocolitis	*Bifidobacteria, Lactobacilli*	(Athalye-Jape et al., 2018; Dermyshi et al., 2017)
Lower abdominal pain in children	*Lactobacillus reuteri* DSM 17938	(Carroll, 2020; Weizman et al., 2016)
Prevention of *C. difficile* infections in hospitalised patients	*Saccharomyces boulardii*	(Pillai & Nelson, 2008; Shen et al., 2017)
***Helicobacter pylori* eradication**	*Lactobacillus reuteri*	(Dore et al., 2016; Lü et al., 2016; Wang et al., 2017)
Caries and periodontitis	*Lactobacillus reuteri*	(Gruner et al., 2016; Martin-Cabezas et al., 2016)
Decreased antibiotic-associated diarrhea	*Lactobacillus acidophilus, Lactobacillus casei*	(Gao et al., 2010; Lönnermark et al., 2010)
Treatment for depression and anxiety	*Lactobacillus helveticus, Bifidobacterium longum*	(Messaoudi et al., 2011; Ohland et al., 2013)
Reduction in allergies	*Lactobacillus gasseri*	(Chen et al., 2010)

When selecting a probiotic product targeting GIT, certain criteria have to be considered. To start, the probiotic has to be a member of a safe species (Barlow et al., 2007). It is essential that the probiotic has a documented beneficial health effect and no adverse side effects. The major challenge for a probiotic is that it must be able to withstand the undesirable conditions in the GIT to reach the desired destination (Figure 1). To start with, the enzymes in the mouth can act as antimicrobial agent (e.g. lysozyme) (Mosca & Chen, 2016; Vila et al., 2019), followed by mechanical stress due to peristalsis and bicarbonate secretion (which establishes a pH gradient that might affect the structure and function of the cell envelope) in the oesophagus (Genova et al., 2019; Papadimitriou et al., 2016). Next, transiting through the stomach—with gastric acids and low pH of 2.0 (Castro-López et al., 2022; De Melo Pereira et al., 2018)—represents a remarkable challenge for probiotic survival. After the stomach, the probiotic enters the intestinal phase, where they need to adapt to a sudden change in pH from highly acidic to neutral. Here, the

probiotic encounters bile salts, pancreatic juice, and proteolytic enzymes (Castro-López et al., 2022; De Melo Pereira et al., 2018). For the microorganism to establish itself in the intestinal microbial community, it should be able to attach to the intestinal lining (De Melo Pereira et al., 2018). In summary, the probiotic needs not only to be alive when administered, it also needs to have high tolerance to several adverse environmental conditions.

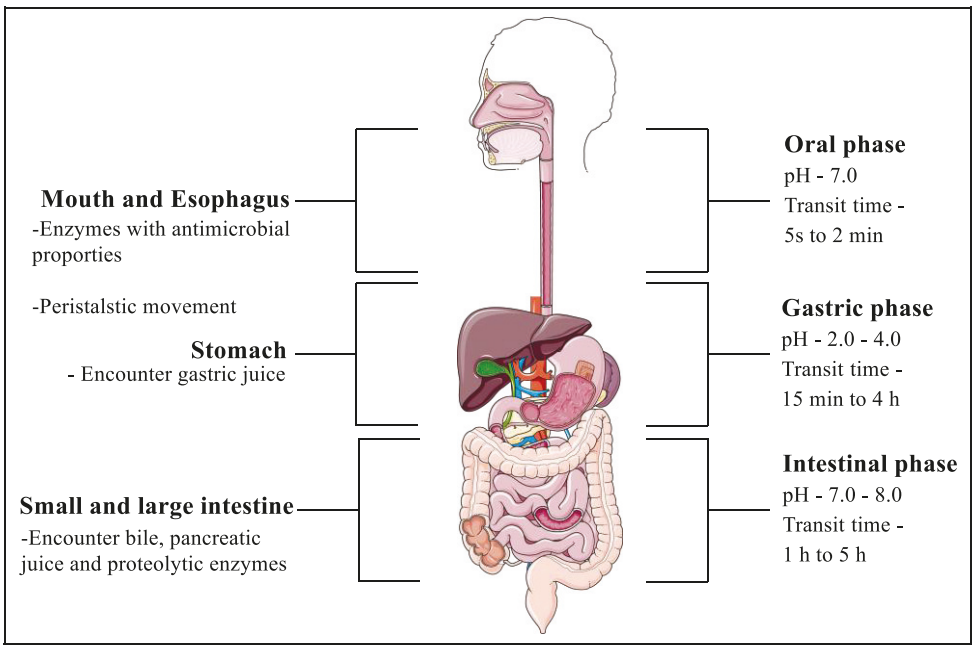


Figure 1. The passage of probiotics through the gastro-intestinal tract (GIT)[2].

The quality of probiotics in commercial products is evaluated based on number of the viable cells. As a rule of thumb, a survival of $\geq 10^8$ CFU/g viable cells is necessary to confer health benefits to the host, but this number depends on bacterial species and indication (Indian Council of Medical Research & Department of Biotechnology, 2011; Minelli & Benini, 2008).

Production of probiotics

Industrial production of probiotic bacteria must be specifically designed to achieve a stable product that can withstand unfavourable environmental conditions during

[2] Digestive-system icon by Servier https://smart.servier.com/ is licensed under CC-BY 3.0 https://creativecommons.org/licenses/by/3.0/

storage while also preserving its probiotic properties (Papadimitriou et al., 2015; Soccol et al., 2010; Van de Guchte et al., 2002). For industrial production, qualities such as high biomass yield, freeze-drying (FD) tolerance, shelf life, and oxygen tolerance are important.

Figure 2. Simplified overview of the production process of probiotics.

Manufacturing consists of several steps in addition to the fermentation step. Figure 2 depicts a simple overview of the production process of probiotics. The first step is fermentation, which includes media and inoculum preparation. Main components of the media are nitrogen sources, carbohydrates, salts, and necessary micronutrients. A cryo-cell bank that has been carefully created and verified to be free of contaminants is used to generate the inoculum by growing it in the desired medium. The fermentation is performed in a bioreactor with controlled parameters for temperature, pH, aeration, and agitation levels. The objective of this step is primarily to obtain a high density of cells with high cell viability. At this step, it is also possible to optimise the robustness of cells to subsequent treatments. After fermentation, the cells are harvested and concentrated via a centrifugation process. The concentrated cells are then prepared according to the final product application. In the pre-formulation step, lyoprotectants are added to protect cells from injury during freezing and/or FD to maintain the cells' structure and composition in the freeze-dried matrix (Santivarangkna et al., 2008). FD is a process in which the water in the sample is frozen into ice and subsequently removed under vacuum (Geidobler & Winter, 2013). FD is used for long-term preservation of cells. For FD to be successful, it is essential that the cells are viable, which allows them to be reactivated when needed. After FD, the probiotic product obtained can be presented in a dried end-product. This freeze-dried product needs to have high probiotic functionality, which depends on the properties of the strain used (e.g., for high tolerance to bile, acid stress, immunomodulation) (Dunne et al., 2001; Iaconelli et al., 2015; Morelli, 2007; Saarela et al., 2000).

Scope of the thesis

The overall objective of the work presented in this thesis was to understand cell responses to critical process parameters during production that have a significant impact on the stability and probiotic functionality of freeze-dried *Limosilactobacillus reuteri*. Parameters that could be altered during fermentation process and that impact cell physiology were studied. In each of the studies, a particular aspect of the process was studied to obtain a probiotic product with high tolerance to FD stress.

In **Paper I**, a study is described in which process parameters, such as temperature, pH, and oxygen, were altered during the cultivation of *L. reuteri* resulting in variations in cell growth, morphology, and FD survivability. A flow cytometric approach was used to investigate the growth and morphology of the microorganism, and correlations between the process parameters and growth and morphology were made. Morphology markers such as forward-scatter (FSC), side-scatter (SSC), and pulse width were used in flow cytometry (FCM) to predict optimal growth and FD stress. Cell morphological heterogeneity was also investigated in this study.

In **Paper II**, the impact of oxygen on the physiology of *L. reuteri* is described. Although oxygen impairs cell growth by radical formation, it may help as a redox sink at the right level. For some species, oxygen is beneficial for FD tolerance as it increases membrane fluidity by incorporating more unsaturated fatty acids (UFA), due to the activity of oxygen-dependent desaturases (such as *Bacillus* and *Escherichia*). However, *L. reuteri* has no annotation of desaturases. In this study, an online FCM was set up along with an automated gating strategy to obtain a detailed picture of cell growth, viability, and morphology. The impact of oxygen on lipid composition was investigated, and its correlation to FD tolerance was studied.

In **Paper III**, the impact of media components on lipid composition was studied. A defined mineral media was established by modifying a previously available semi-defined media. The effect of external fatty acids source Tween 80 on growth, lipid composition, and FD tolerance was studied.

In **Paper IV**, the impact of time spent for pre-formulation step on FD tolerance and long-term storage was examined. Immediate freezing, freezing after 3h and freezing after 3h with additional lyoprotectant added, were the test cases. Growth, and metabolism were recorded for 3h. After FD, an accelerated shelf-life study was conducted to understand the implications of the pre-formulation hold time.

2. Physiology of *Limosilactobacillus reuteri*

The genus *Lactobacillus* was first mentioned by Beijerinck in 1901. The genus *Lactobacillus* belongs to the *Lactobacillaceae*, which also contains the other genera *Paralactobacillus* and *Pediococcus*. Classification of lactobacilli was initially determined by considering phenotypic features such as the optimal temperature for growth, carbohydrate utilisation, and the range of metabolites produced (Orla-Jensen, 1919). In the latter part of the 20th century, genotypic and chemotaxonomic criteria such as DNA–DNA hybridisation, the mol% G+C content, and the peptidoglycan's chemical structure were used to distinguish new bacterial species. Comparison of the 16S ribosomal RNA of different organisms make it possible to resolve evolutionary relationships and create phylogenetic schemes to classify and name bacteria (Hugenholtz, 2002). Greater accessibility to whole genome sequencing over the past 15 years has facilitated the sequencing of entire bacterial genomes. Average nucleotide identity (ANI) values of genes shared between two bacterial genomes have become the top standard for identifying new bacterial species. There were only 36 identified *Lactobacillus* species by 1980, but this number rose to 261 by 2020 with the advent of these techniques (Biogaia AB, n.d.; Zheng et al., 2020).

Limosilactobacillus reuteri, previously known as *Lactobacillus reuteri* is a gram-positive bacterium (Zheng et al., 2020). It was first isolated in 1962 by the microbiologist Gerhard Reuter, but it was initially classified as *L. fermentum biotype II*. It was not until 1980 that Otto Kandler and his group described it as a separate species and named it after Reuter who first isolated it (Kandler et al., 1980). In 2020, the genus *Lactobacillus* was reclassified into 25 genera. *Lactobacillus reuteri*, which belonged to genus *Lactobacillus,* was classified into the new genus *Limosilactobacillus*. The word 'limosus' means 'slimy' in Latin and *Limosilactobacillus* is named as such due to the production of exopolysaccharides (EPSs) from sucrose by all species in the genus (Zheng et al., 2020). *L. reuteri* is a heterofermentative lactobacilli which produces lactate, acetate, ethanol, and carbon dioxide via the phosphoketolase pathway (PKP) and Embden–Meyerhof–Parnas (EMP) pathway. It is facultatively anaerobic, catalase- negative, and non-spore-forming. Optimal growth is observed at 37 °C for most of the species belonging to this genus (> 15 °C and < 45°C). *L. reuteri* and related species within the genus

Limosilactobacillus generate exopolysaccharides from sucrose, which facilitate the formation of biofilms across the non-secretory epithelia of the GIT (Walter et al., 2008; Zheng et al., 2015). The genome size of the type of strain is 1.94 Mbp. The mol% G+C content of DNA is 38.6 (Zheng et al., 2020). The strain used in this thesis *L. reuteri* ATCC 55730 (mother strain of DSM 17938), was isolated from the breast milk of a woman in South America (Saulnier et al., 2011). *L. reuteri* DSM 17938 is a well-studied probiotic bacterium that can be used to target human conditions such as abdominal pain, diarrhoea (acute, infectious, and nosocomial), infant colic, and necrotising enterocolitis (Chau et al., 2015; Dinleyici et al., 2015; Francavilla et al., 2012; Greifová et al., 2017; Liu et al., 2019; Oncel et al., 2014; Roos et al., 2013; Wanke & Szajewska, 2012; Weizman et al., 2016).

Nutrient requirements and growth conditions

An understanding of the natural environment in which a microorganism lives can be beneficial for selecting an appropriate culture medium as the nutrient needs of the microorganism are reflective of its native habitat (Willey et al., 2022). The medium employed in the cultivation of bacteria is usually composed of a complex mix of nutrient-rich components to allow for fast growth. De Man-Rogosa-Sharpe (MRS) medium has been used extensively for the cultivation of lactic acid bacteria) LAB, including *L. reuteri* (De Man et al., 1960). MRS contains sources of carbon, nitrogen, salts, and a buffer to maintain pH. Glucose is often used as the carbon source. Enzymatic digests of yeast and beef extract are complex mixtures of organic molecules such as peptides, amino acids, lipids, and vitamins. Sodium acetate and ammonium citrate suppresses the growth of competing bacteria (Todorov & Dicks, 2009). Potassium phosphate is the pH buffering agent. Tween 80 is a surfactant used as a growth supplement because it is a fatty acid source as well as help to solubilise (e.g., solubilise fats). LAB can also be cultivated in a defined mineral medium (Paper III) where all ingredients are known to understand the metabolism of the bacteria (Table 2).

The biosynthesis of amino acids requires a significant amount of energy. The energy used should be reduced when the goal of the fermentation is to maximise cell mass by incorporating the amino acids in the medium. Conversely, LAB are unable to proliferate without the addition of exogenous amino acids in the absence of mineral nitrogen (Van Niel & Hahn-Hägerdal, 1999). LAB species have reported different amino acid requirements (Kwoji et al., 2022; Saguir & De Nadra, 2007); this may be due to the absence of functional specific biosynthetic genes or the presence of specific regulatory mechanisms (Chopin, 1993; Saulnier et al., 2011). Fourteen amino acids were found to be essential for the growth of *L. reuteri* DSM 17938 (Paper III). Vitamins of the B group are essential for LAB. Nicotinate is necessary for the synthesis of NAD(P), pantothenate is the cofactor

of CoA, biotin is required for oleic acid synthesis, pyridoxal is involved in synthesis of amino acids, riboflavin is necessary for the cofactor FAD, folate is required for purine synthesis, and thiamine is a cofactor essential for glycolysis, amino acid synthesis, and nucleotide metabolism (Cocaign-Bousquet et al., 1995; Costliow & Degnan, 2017).

Table 2. Media components in defined mineral medium (DMM1). (Paper III)

Ingredients	
Carbon	Glucose
Nitrogen	Ammonium citrate, Alanine, Arginine, Cysteine, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Methionine, Phenylalanine, Serine, Tyrosine, Tryptophan, and Valine
Vitamins	Biotin, Pyridoxal-HCl, Folic acid, Riboflavin, Nicotinic acid, Thiamine-HCl, Pantothenate, and *p*-Aminobenzoic acid
Nucleotides	Guanine, Adenine, and Uracil
Minerals	Zinc, Manganese, Cobalt, Molybdenum, Calcium, Copper, Iron, Potassium, and Boron
Reducing agent	Reduced glutathione

In addition to the nutrients required, it is important to notice the growth conditions of the bacteria as they play a key role in proliferation. Temperature and pH both impact growth. Bacteria exhibit a broad spectrum of pH tolerance, but they are not without limits. A low external pH means a higher concentration of H^+ ions outside the cell, leading to reduction of cytoplasmic pH. Such perturbations can pose a significant threat to bacterial viability, manifesting in disrupted plasma membrane integrity and inhibition of enzymatic and membrane transport processes. Moreover, shifts in external pH can modify the ionisation of nutrient molecules, thereby limiting their bioavailability to the microorganism (Willey et al., 2022). The impact of temperature on growth is a crucial consideration that is primarily determined by the temperature sensitivity of enzymatic catalysis and membrane function. Enzymes operate optimally at specific temperatures, beyond which their catalytic activity is lost (Willey et al., 2022). *Lactobacillus sps.* grow at an optimal pH between 5.0–6.0 and temperatures between 30 °C–40 °C (Liu et al., 2014; Schoug et al., 2008). Cultivating bacteria in sub-optimal conditions may result in lowered growth, which would act as a stress factor for the cell (Fernández Murga et al., 2000; Hernández et al., 2019; Liu et al., 2014; Lorca & De Valdez, 1999; Wang et al., 2005); this may ultimately influence the bacterial tolerance to subsequent adverse environments. I found that low temperature during growth was beneficial for FD stress tolerance of *L. reuteri* (Paper I).

Morphology

Lactobacillus sps. are rod-shaped bacteria ranging from 1–3 μm in size. Growth conditions define the morphology of the cells. Researchers have previously observed that growing LAB in different pH levels, culture media, and temperatures induces changes in their morphology (Palmfeldt & Hahn-Hagerdal, 2000; Rao et al., 2021; Senz et al., 2015; Šušković et al., 2000). They are known to have a pleomorphic behaviour (Figure 3). Morphological heterogeneities were observed under non-optimal growth conditions, with low temperature, high initial pH, and high oxygen levels triggering changes in morphology towards cell chain formation (Paper I). Cell morphology and number of cells per chain are controlled by several regulatory mechanisms governing cell fission and separation of cells in later stages of the cell cycle. It is unclear which parameters rule the distribution of cell size, chain length, and number of cells per chain (Weart *et al.*, 2007; Van Bokhorst-van de Veen *et al.*, 2011). It can be speculated that cells connected to each other in a chain will be able to communicate with each other and would share a similar fate as a response to changes in external environment, but the mechanism behind this remains unclear.

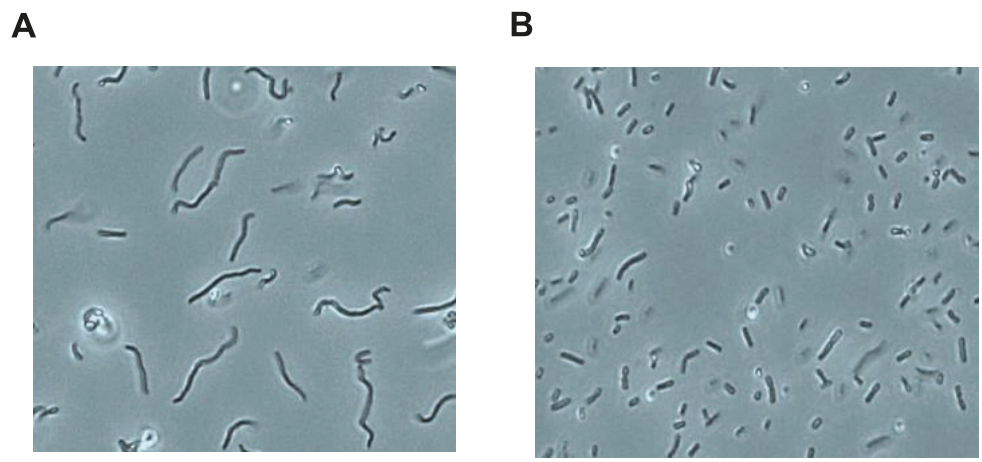


Figure 3: Light Microscopy imaging (100X) of *L. reuteri* cultivated at (A) 30 °C, and (B) 37 °C. *(*Adapted from Paper I)

Central carbon metabolism

LAB employ different glycolytic pathways to metabolise carbohydrates (mono-, disaccharides) into the common, three-carbon intermediates of the glycolysis pathway (dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, pyruvate, etc.) (Burgé et al., 2015). Homofermentative LAB utilise the Embden–Meyerhof–Parnas (EMP) pathway or pentose phosphate pathway, whereas, heterofermentative LAB utilise the phosphoketolase pathway (PKP) to assimilate pentoses and hexoses (Gänzle, 2015).

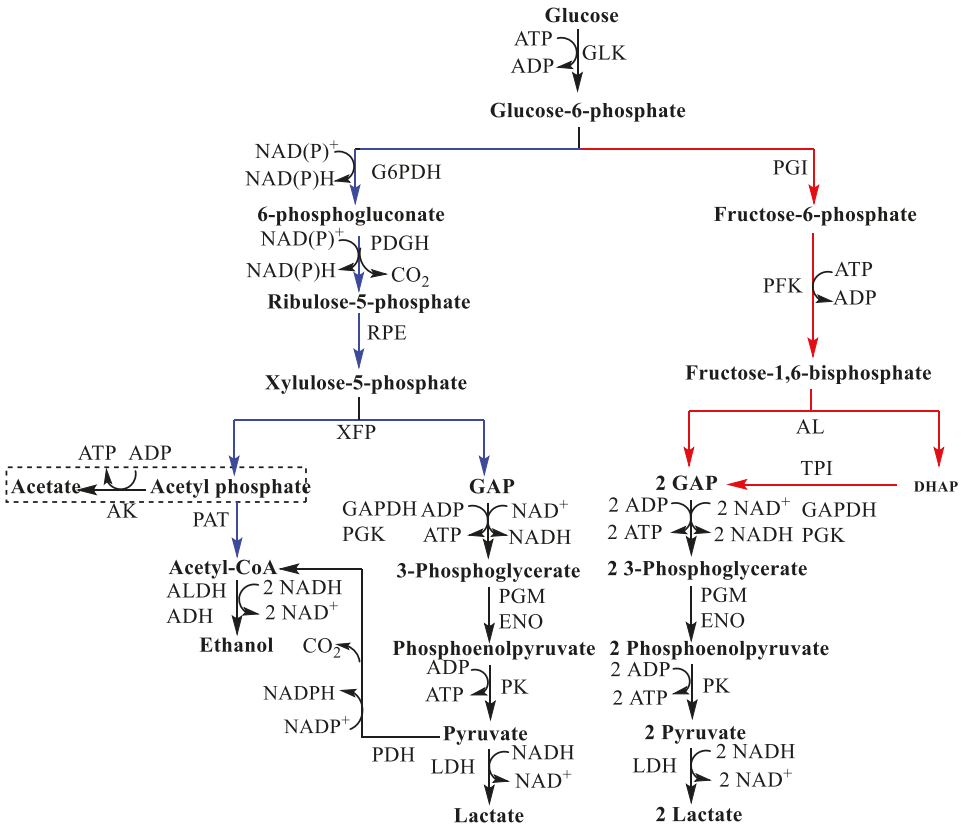


Figure 4. Pathway of glucose metabolism in *L. reuteri* DSM 17938. Phosphoketolase pathway (PKP) presented in blue, Embden–Meyerhof–Parnas pathway (EMP) presented in red, and common reactions represented in black in both pathways. GLK, glucokinase; G6PDH, glucose-6-phosphate dehydrogenase; PGI, Phosphoglucose isomerase; PFK, Phosphofructose kinase; PDGH, 6-phosphogluconate dehydrogenase; RPE, ribulose phosphate 4-epimerase; XFP, Xylulose 5-phosphate phosphoketolase; AL, Aldolase; TPI, Triose phosphate isomerase; GAP, GAPDH, Glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PAT, phosphate acetyltransferase; ALDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; AK, acetate kinase; PDH, pyruvate dehydrogenase. Reaction in the dashed box occurs in presence of an electron acceptor.

Heterolactic LAB assimilate glucose to produce lactate, acetate, ethanol, and CO_2 via the PKP (Årsköld et al., 2008; Kandler, 1983) (Figure 4). *L. reuteri* DSM 17938 has previously been shown to utilise both the EMP pathway as well as the PKP (Årsköld et al., 2008; Saulnier et al., 2011). The PKP has a lower yield of ATP per glucose molecule compared to that of the EMP (2 ATP for the EMP and 1 ATP for the PKP). However, Årsköld et al. found that *L. reuteri* prefers the PKP, whereas the EMP pathway is used as a shunt to provide energy.

LAB are able to assimilate a number of different sugars and produce reduced fermentation products (Table 3). Depending on the sugar type, a particular transporter (either by phosphoenolpyruvate-dependent phosphotransferase system, by permease, or by symport/antiport system) is activated that facilitates the transport of sugar into the cell. Saulnier et al. (2011), displayed that *L. reuteri* ATCC 55730 (the mother strain of DSM 17938 and the strain used throughout the thesis) was capable of catabolising a range of different carbon sources such as glucose, galactose, lactose, raffinose, lactulose, maltose, and galacto-oligosaccharides (GOSs).

Table 3. Substrates and products during fermentation.

Substrate	Product	Metabolic mode of action	References
Monosaccharides			
Glucose	Lactate, Ethanol and CO_2	PKP and EMP	(Burgé et al., 2015) Paper I, II, III & IV
Galactose	Lactate, Acetate, Ethanol and CO_2	Leloir pathway, PKP and EMP	(Neves et al., 2010; Zeng et al., 2010)
Disaccharides and trisaccharides			
Sucrose (Glucose + Fructose)	Lactate, Acetate, Ethanol, Mannitol, CO_2, and EPS	PKP and EMP	(Årsköld et al., 2008)
Lactose (Glucose + Galactose)	Lactate, Acetate, Ethanol and CO_2	Leloir pathway, Tagatose pathway, EMP and PKP	(Zeng et al., 2010)
Maltose (Glucose + Glucose)	Lactate, Ethanol and CO_2	PKP and EMP	(Zhao & Gänzle, 2018)

Electron acceptors

An electron acceptor is a molecule capable of accepting or receiving one or more electrons from another molecule or atom, thereby becoming reduced in the process. In chemical reactions, electron acceptors are typically paired with electron donors, which are the molecules or atoms that provide the electrons. When an electron donor transfers one or more electrons to an electron acceptor, it becomes oxidised. For

example, during lactic acid fermentation, pyruvate is reduced to lactate, and NADH is oxidised to NAD^+. The NAD^+ is then recycled back to the glycolytic pathway to continue the breakdown of glucose. In this process, lactate acts as an electron acceptor, accepting electrons from NADH to generate NAD^+. Without lactate as an electron acceptor, the fermentation process would not be able to continue. Electron acceptors thus play a crucial role in facilitating the fermentation process by accepting electrons from reduced organic compounds and facilitating the production of ATP.

Årsköld et al. (2008) revealed that the ratio between the EMP pathway and PKP is not 1:1. Metabolic flux analysis demonstrated that there is a bias towards the redox-heavy PKP, which accounts for over 70% of the total glucose flux; this is attributed to an ineffective phosphofructokinase (pfk) in the EMP pathway. However, this can be compensated for by the addition of external electron acceptors that create an alternative way to oxidise NAD(P)H, resulting in a gain of 1 ATP in the PKP per oxidation reaction and rendering the PKP as efficient as the EMP pathway. Use of electron acceptors such as fructose has been shown to increase biomass yield and growth rate in LAB (Årsköld et al., 2008; Borch & Molin, 1989; Lucey & Condon, 1986). In the following section, different external electron acceptors are discussed.

Sucrose and fructose

Sucrose is a disaccharide comprising fructose and glucose subunits. Sucrose is converted to glucose-1-phosphate and fructose via the enzyme sucrose phosphorylase. Fructose is an external electron acceptor enabling the microorganisms to restore the redox imbalance and grow at a higher rate compared to glucose alone. In the case of fructose, $NAD(P)^+$ regeneration occurs through the reduction of fructose into mannitol by means of mannitol dehydrogenase (MDH) (Figure 5), resulting in the conversion of acetyl phosphate into acetate and ATP instead of ethanol.

Sucrose has been used as a lyoprotectant in all my studies. In Paper IV, I discussed about the conversion of sucrose to fructose and possibly EPS during pre-formulation hold time which had negative implications on the long-term storage of probiotics.

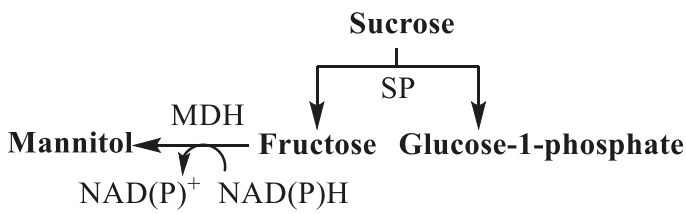


Figure 5. Sucrose conversion. SP – Sucrose phosphorylase, MDH – Mannitol dehydrogenase.

Glycerol

In *L. reuteri*, glycerol can be used as an electron acceptor. *L reuteri* undergo the conversion of glycerol in a specialised bacterial compartment known as metabolosome, which might be attributed to the toxicity associated with the final end chemical compound produced (Sriramulu et al., 2008). The oxidative and ATP-generating branch of the glycerol pathway generates 3-hydroxypropionate from glycerol, whereas the reducing branch regenerates NAD^+ and produces 1,3-propanediol (Dishisha et al., 2014; Gänzle, 2015; Luo et al., 2011) (Figure 6). In fermentation with hexoses and glycerol, only the reducing branch of the pathway is used to support ATP generation from acetyl phosphate (Luo et al., 2011). In addition, the intermediate 3-hydroxypropionaldehyde (3-HPA) is excreted into the medium where it undergoes rapid dimerisation and hydration that results in the formation of HPA-dimer and HPA-hydrate. This complex of hydrated, non-hydrated, and dimeric forms of 3-hydroxypropionaldehyde is called a reuterin system that has antimicrobial activity (Asare et al., 2020; Spinler et al., 2017).

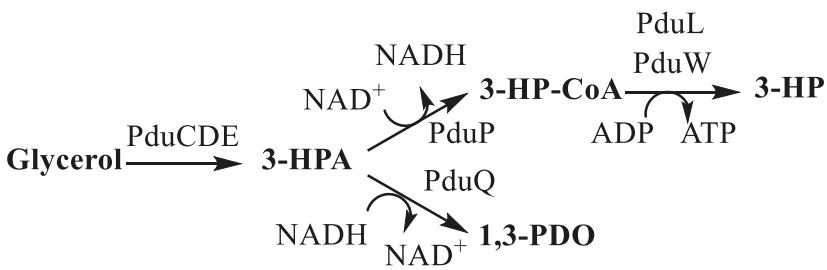


Figure 6. Glycerol utilisation. 3-HPA – 3-hydroxypropionaldehyde; 3-HP-CoA – 3-hydroxypropionyl-CoA; 1,3-PDO – 1,3-propanediol; 3-HP – 3-hydroxypropionate; PduCDE – B12-depdendent glycerol dehydratase; PduP – propionaldehyde dehydrogenase; PduQ – 1,3-propanediol oxidoreductase; PduL – phosphotransacylase, PduW – propionate kinase.

Oxygen

L. reuteri is facultative anaerobic and lacks a functional proton-translocating respiratory chain (Papadimitriou et al., 2016). It can only tolerate oxygen at low levels (Paper I). Nevertheless, oxygen serves as an electron acceptor with high affinity for electrons due to its high electronegativity (Willey et al., 2022). Oxygen may enhance the activities of NADH oxidase (direct enzymatic oxidation of NADH), which compete with lactate dehydrogenase for NADH molecules (De Angelis & Gobbetti, 2004; Papadimitriou et al., 2016) (Figure 7). Thus, the production of lactic acid is reduced, and the glycolytic flux is redirected toward production of acetate (with additional ATP production), ethanol, and CO_2 (Paper II).

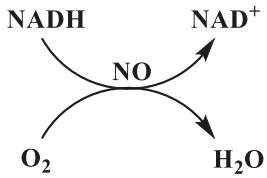


Figure 7. NADH oxidase (NO).

Citrate

Citrate is found in substantial quantities (approximately 2 mM) in human breast milk (Hoppe et al., 1998). Citrate assimilation occurs via the following four steps: (i) uptake of citrate via permease (CitP), (ii) formation of oxaloacetate and acetate from citrate through the activity of citrate lyase (CL), (iii) decarboxylation of oxaloacetate into pyruvate via oxaloacetate decarboxylase (OAD), and (iv) reduction of pyruvate to lactate. The end-products are acetate, succinate, pyruvate, and lactate (Gänzle, 2015; Marty-Teysset et al., 1996; Papadimitriou et al., 2016; Paper III).

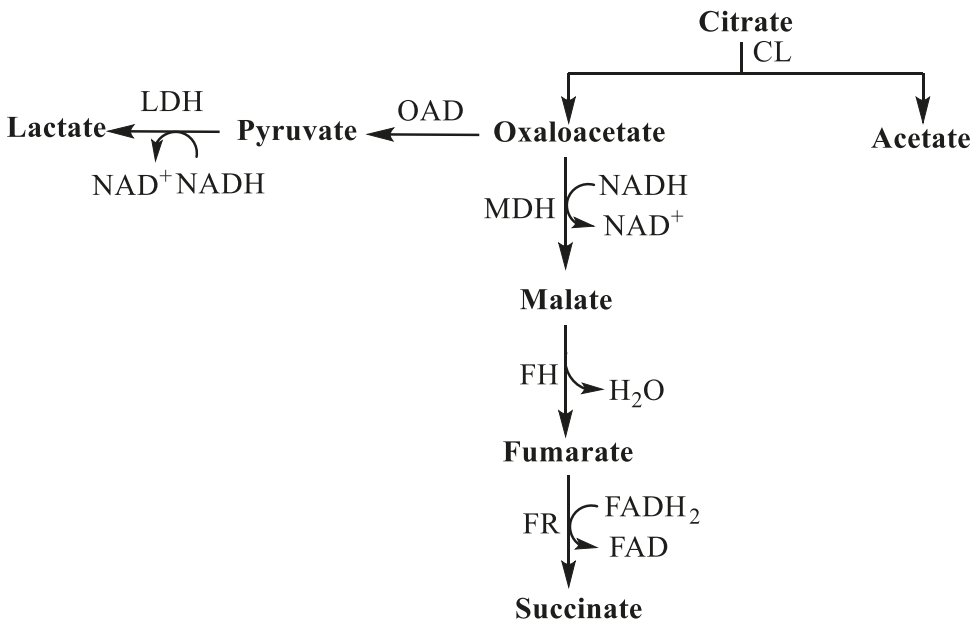


Figure 8. Citrate metabolism in LAB. CL, citrate lyase; MDH, malate dehydrogenase; FH, fumarate hydratase; FR, fumarate reductase; OAD, oxaloacetate decarboxylase; LDH, lactate dehydrogenase.

Citrate is thereby indirectly an electron acceptor since its assimilation results in oxidation of 1–2 moles of NADH per citrate (Figure 8). The citrate present in the medium contributes to increased pyruvate production through two steps of enzymatic reactions (CL and OAD) that cause increased production of lactate and, consequently, increased oxidation of NADH. The re-oxidation of NADH required for ethanol production decreases, which shifts the flux towards formation of acetate from acetyl phosphate via acetate kinase (AK) along with the generation of additional ATP (Figure 4) (Ricciardi et al., 2022). Another route for the assimilation of citrate is via the reductive TCA (tricarboxylic acid) pathway. Previously, researchers have observed formation of malate and succinate during growth in a medium containing citrate. This pathway involves the enzymatic reactions catalysed by citrate lyase, malate dehydrogenase, fumarate hydratase (fumarase), and fumarate reductase.

Studies in *Leuconostoc mesenteroides* (a heterofermentative LAB) have demonstrated that co-metabolism of glucose and citrate results in the production of acetate. Acetate formation is from glucose and the pyruvate pool of citrate. Lactate came from pyruvate of citrate metabolism. Acetate from citrate metabolism was incorporated into biomass, primarily into lipids (Schmitt et al., 1992); this was studied using radio-labelled glucose and citrate and performing enzymatic assays. During anaerobic cultivation using MRS medium, there was no citrate assimilation in *L. reuteri* (Paper II), whereas cultivation using defined mineral medium displayed assimilation of citrate and formation of succinate (Paper III). From these results, I can speculate that citrate assimilation in *L. reuteri* depend on the medium composition.

Lipids in LAB

Lipid composition in LAB plays a vital role in stress tolerance and varies according to environmental conditions (Fonseca et al., 2019; Hua et al., 2009; Li et al., 2009; Liu et al., 2014; Schoug et al., 2008; Taranto et al., 2003). Most bacteria employ a type II fatty acid synthase (FASII) system (Zhang & Rock, 2016) (Figure 9).

Fatty acid biosynthesis

The biosynthesis of fatty acids (FAs) is tightly regulated and consists of three stages: (i) initiation, (ii) elongation, and (iii) termination and transfer into the bilayer. In the initiation stage, the acetyl-CoA is converted into malonyl-ACP by acetyl-CoA carboxylase (ACC) and malonyl-CoA:ACP transacylase (FabD; Figure 9). The 3-oxoacyl-ACP synthase III (FabH) enzyme catalyses the condensation of acetyl-CoA with malonyl-ACP, resulting in acetoacetyl-ACP. The elongation of fatty acid

chains is an iterative process that involves 2-carbon building blocks. The 3-oxo-acyl-ACP intermediate is reduced by the 3-oxoacyl-ACP reductase (FabG), and the 3-hydroxyacyl-ACP is dehydrated into trans-2-enoyl-ACP by 3-hydroxyacyl-ACP dehydratases (FabA or FabZ). Each cycle is led to completion by an enoyl-ACP reductase (FabI) that reduces the double-bond in trans-2-enoyl-ACP using the cofactor NADH. In the termination step, a mature length acyl moiety is driven towards phospholipid synthesis enzymes. The acyl chains of fatty acids are mainly composed of an even number of carbons ranging from 12 to 22, involving either zero or one to three unsaturations (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C22:0), (Fonseca et al., 2019; Paper II).

Long-chain acyl-ACP are introduced into the membrane via the PlsX-PlsY pathway. Phosphate acyltransferase (PlsX) mediates the transfer of the acyl group from the acyl-ACP complex into acyl phosphate, which is used by glycerol-3-phosphate acyltransferase (PlsY) to convert glycerol-3-phosphate into lysophosphatidic acid (LPA; Figure 10). Acyltransferase (PlsC) acylates the LPA to form phosphatidic acid (PA), the key intermediate in the formation of membrane phospholipids. PA, along with cytosine triphosphate (CTP), gives rise to cytosine diphosphate (CDP)-diacylglycerol (CDP-DAG). In the next step, glycerol-3-phosphate is exchanged with CMP to form phosphatidylglycerol phosphate (PGP). The final steps leading to the formation of phosphatidylglycerol (PG) involve the dephosphorylation of PGP. PG is further converted into cardiolipin via condensation of two PG molecules by cardiolipin synthase (Cls). PG also forms lysylphosphatidylglycerol (LPG), a major component of the bacterial membrane with a positive net charge mediated by phosphatidylglycerol lysyltransferase (LPGS) (Uniprot, 2023). According to Saulnier et al. (2011), PG, LPG, and cardiolipin are incorporated into the biomass (representing 68%, 23%, and 9% of the total phospholipids, respectively).

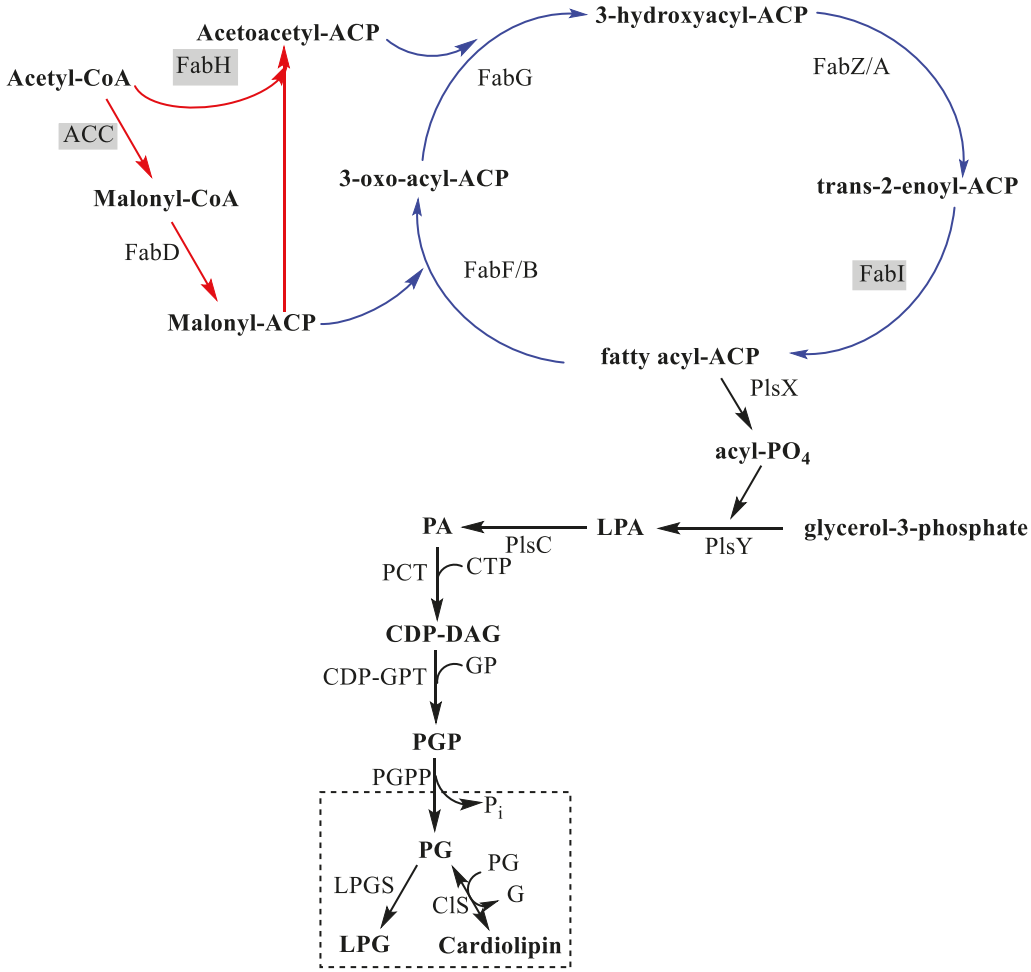


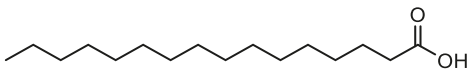
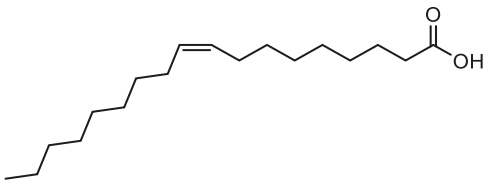
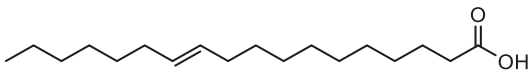
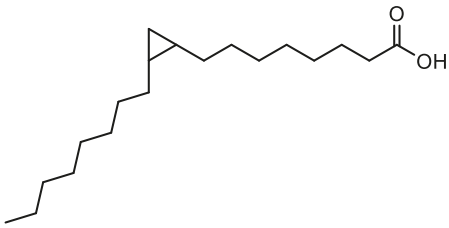
Figure 9. Fatty acid biosynthesis, including phospholipid biosynthesis. Fatty acid biosynthesis occurs in three steps: (i) initiation (red), (ii) elongation (blue), and (iii) termination and transfer into the bilayer. The long-chain acyl-ACP end-products exert feedback regulation over three enzymes, ACC, FabH, and FabI (shown on gray squares). The dashed box represents that these phospholipids can be incorporated into biomass. Drawn with information from Geiger (2019), Saulnier et al. (2011) and Zhang and Rock (2008). ACC - acetyl-CoA carboxylase; FabD - malonyl-CoA:ACP transacylase; FabH - 3-oxoacyl-ACP synthase III; FabF - 3-oxoacyl-ACP-synthase II; FabB - 3-oxoacyl-ACP-synthase I; FabG - 3-oxoacyl-ACP reductase; FabZ/A - 3-hydroxyacyl-ACP dehydratases; FabI - enoyl-ACP reductase; PlsX - Phosphate acyltransferase; PlsY - Glycerol-3-phosphate acyltransferase; PlsC - LPA - lysophosphatidic acid; PA - phosphatidic acid; CPT - cytosine triphosphate; PCT - Phosphatidate cytidylyltransferase; GP - glycerol-3-phosphate; CDP-GPT - CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase; PGP - phosphatidylglycerol phosphate; PGPP - phosphatidylglycerol phosphate phosphatase; PG - phosphatidylglycerol; G - glycerol; ClS - cardiolipin synthase; LPGS - Phosphatidylglycerol lysyltransferase; LPG - lysylphosphatidylglycerol.

Bacteria possess multiple enzymes that can quickly respond to environmental shifts by reorganising the composition of existing phospholipids. The arrangement of fatty

acid chain structure affects the biophysical characteristics of the membrane bilayer as well as the biological significance (Fonseca et al., 2019; Zhang & Rock, 2008). Fatty acids of 16 and 18 carbons account for more than 60% of the total FA of LAB membranes. Regulation of membrane fluidity has been shown to counter stress in LAB. Saturated fatty acid (SFA) are formed as a part of FA biosynthesis (Table 4). Oxygen-dependent desaturases (e.g., *desA*) introduce a *cis* double-bond into pre-existing fatty acids and are responsible for the formation of *cis*-UFA. To adapt to environmental challenges, few bacteria have the capability to convert their *cis*-UFA to *trans*-UFA via the action of the cis-trans isomerase enzyme (CTI); *Trans* UFA enables membranes to be tightly packed, more rigid, and more resistant to solvents (Zhang & Rock, 2008). Cyclic fatty acids (CFAs) are commonly found in LAB and are formed via the methylation reaction carried out by cyclopropane fatty acid synthase (CFS). CFAs have similar biophysical properties to the lipid organisation that are composed of *cis*-UFAs as the cyclopropane bond retains the *cis*-double bond configuration (Grogan & Cronan, 1997). Some bacteria are unable to synthesise UFAs through the action of desaturase enzymes. Instead, they utilise alternate pathways to produce UFAs. One such pathway involves the enzyme FabA, which functions as both a dehydratase and an isomerase. In the absence of desaturase enzymes, FabA hinders the reduction by FabI since the *cis* isomer is not accepted as a substrate. This isomerisation reaction creates a double-bond in the fatty acid chain, producing a UFA (Heath & Rock, 1996; Keweloh & Heipieper, 1996). Another pathway involves the use of exogenous fatty acids. Desaturase-negative bacteria and desaturase-positive bacteria are able to uptake and incorporate UFAs from their environment (Corcoran et al., 2007; Partanen et al., 2001; Tan et al., 2012). These exogenous fatty acids can then be used to meet the bacterial cell's requirements for UFAs.

In the case of *L. reuteri*, there was no indication of the presence of a fatty acid desaturase (*desA*) based on the current gene annotations and the protein domain analysis (Paper II). Uncertainty remains regarding how the UFA are formed in *L. reuteri*. Paper III highlights the impact of adding an exogenous fatty acid source on the lipid composition. Lipid composition of cells cultivated in DMM1 exhibited high degree of SFA which were significantly different to DMM1 supplemented with Tween 80. The levels of UFA produced were very low and there were no differences in UFA profiles between the two mediums. This suggests *L. reuteri* prefers producing SFA by *de novo* synthesis, although further investigation is needed.

Table 4: Regulation of membrane biophysical properties.

Fatty acid	Structure	Enzymes	Effect on membrane fluidity
SFA		Fatty acid biosynthesis	Decreased
***Cis*-UFA**		Desaturase, Fab A	Increased
***Trans*-UFA**		CTI	Decreased
Cyclic FA		CFS	Increased

3. Methods for monitoring fermentation progress

Output variables during and after fermentation

As the cell proliferates, its physiological state changes dynamically; this needs to be monitored in a systematic manner using process analytical technology (PAT) tools to understand the correlation between the input variables (process parameters) and the output variables (Figure 10).

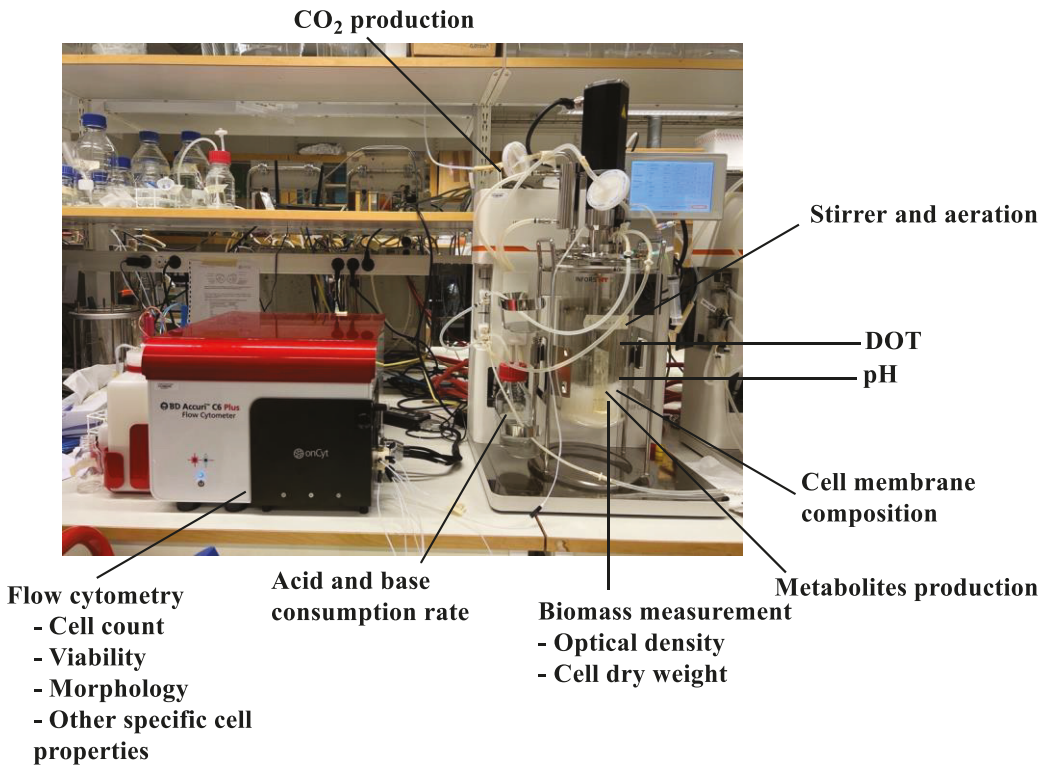


Figure 10: Output variables during and after fermentation that can be analysed.

PAT tools refer to a set of measurement, analysis, and control techniques that are used to monitor and control critical process parameters in real-time during production (Glassey et al., 2011). These tools are used to monitor various process parameters such as temperature, pH, dissolved oxygen, cell growth, and product quality attributes. By providing real-time feedback, PAT tools enable users to adjust the process immediately, leading to more consistent and high-quality data and products.

Bacterial growth can be monitored in many ways during fermentation. The most common way of determining growth is either by direct or indirect methods. Direct methods of growth include optical density measurements (Stevenson et al., 2016), which is a non-invasive technique that involves quantifying microbial growth by measuring the absorption of light using cells present in the fermentation broth with online sensor spectrophotometry. Cell dry weight analysis is another method to monitor growth by measuring the amount of biomass produced (De Vrije et al., 2007), and the flow cytometry (FCM) approach can also be employed. Base consumption, which is measured to maintain pH, is an indirect measure of growth. Additionally, CO_2 production can provide information regarding the growth stages of microorganisms.

Cell viability is a measure of the proportion of live, healthy cells within a population. Assay for cell viability can be either culture-dependent or culture-independent. The most common way of assessing cell viability is to perform a plate count enumeration, whereby the sample is plated on agar plates via serial dilution technique. Other methods include most probable number (MPN) and isothermal calorimetry (Garcia et al., 2017). Culture-independent methods are more rapid. FCM analysis utilising fluorescent stains (e.g., CFDA-SE/PI, SYTO9/PI) was used to distinguish between live and dead cells (Zotta et al., 2012; Paper III & IV).

Sugars and metabolites can be measured using high performance liquid chromatography (HPLC) and gas chromatography (GC). Rates and yields of substrate consumption and metabolite formation can be calculated. Rates and yields depend on whether cells are grown in sub-optimal conditions or exposed to stress factors. Specific enzyme activity (e.g., lactate dehydrogenase) can be assayed to understand their product formation rate in the biosynthetic pathways of the bacteria. Morphology can be monitored using microscopes or by using FCM (Paper I); this provides information on cell size, cells per chain, and if there is any aggregation. Expression of genes can be understood by performing transcriptomics (mRNA level) or proteomics (protein level).

There are numerous observed variations in LAB cells, and employing these methods may offer insight into a few of their mechanisms of action. Traditional analysis of microbial cultures is performed using averaged cell characteristics from samples containing millions of cells and therefore disregards heterogeneity among the population (Heins et al., 2019). Microbial cultures used in bioprocesses are often of

the same genotype, and cell heterogeneity can be minimised if the environmental parameters are maintained within the optimal range (Lencastre Fernandes et al., 2011). However, bacteria exhibit intercellular heterogeneity that is impacted by environmental conditions, which then also contributes to the overall process performance (Delvigne et al., 2017; Lemoine et al., 2017; Müller & Nebe-Von-Caron, 2010). Heterogeneity can play a significant role in bacterial fermentation by enhancing substrate utilisation, improving product quality, and increasing robustness. Heterogeneity can be measured by experimental methods at the single-cell level (Hare et al., 2021; Lencastre Fernandes et al., 2011). The chapter discusses more in detail about the flow cytometric approach.

Flow cytometry

FCM is a tool used to analyse the physical and chemical characteristics of particles suspended in a fluid medium. Throughput and analysis time are improved when compared to fluorescence microscopy and other fluorescence-based assays such as fluorescence in situ hybridisation (FISH) and enzyme-linked immunosorbent assay (ELISA). FCM is a culture-independent technique that is fast (seconds to minutes) and produces accurate and reproducible outcomes. Its speed, sensitivity, and versatility make it a powerful tool for the analysis of cells and particles.

Principle

A basic flow cytometer consists of a few essential components, including a light source (arc lamps and lasers), a flow cell, a hydraulic fluidic system, optical filters to select desired wavelengths, photomultiplier tubes to register the desired signals, and a data processing unit (Figure 11). Hydrodynamic focusing in the fluid stream with sheath fluid as the carrier enables analysis of a single cell. At the interrogation point, the cells/particles are illuminated by the light source (arc lamps and especially lasers), depending on the type of cell analysis. Modern equipment uses a wide variety of lasers which emit light of different wavelengths. The light that is scattered or emitted from the cells or fluorescent molecules bound to the cells can be isolated and optically separated using collection optics, mirrors, and filters. The light scattered in the direction of the incident light (0–10°) is known as FSC and gives information on the size, whereas the light scattered perpendicular (90°) to the incident light is known as the SSC and gives information on the granularity of the cell (Muirhead et al., 1985; Shapiro, 2003) (Figure 11).

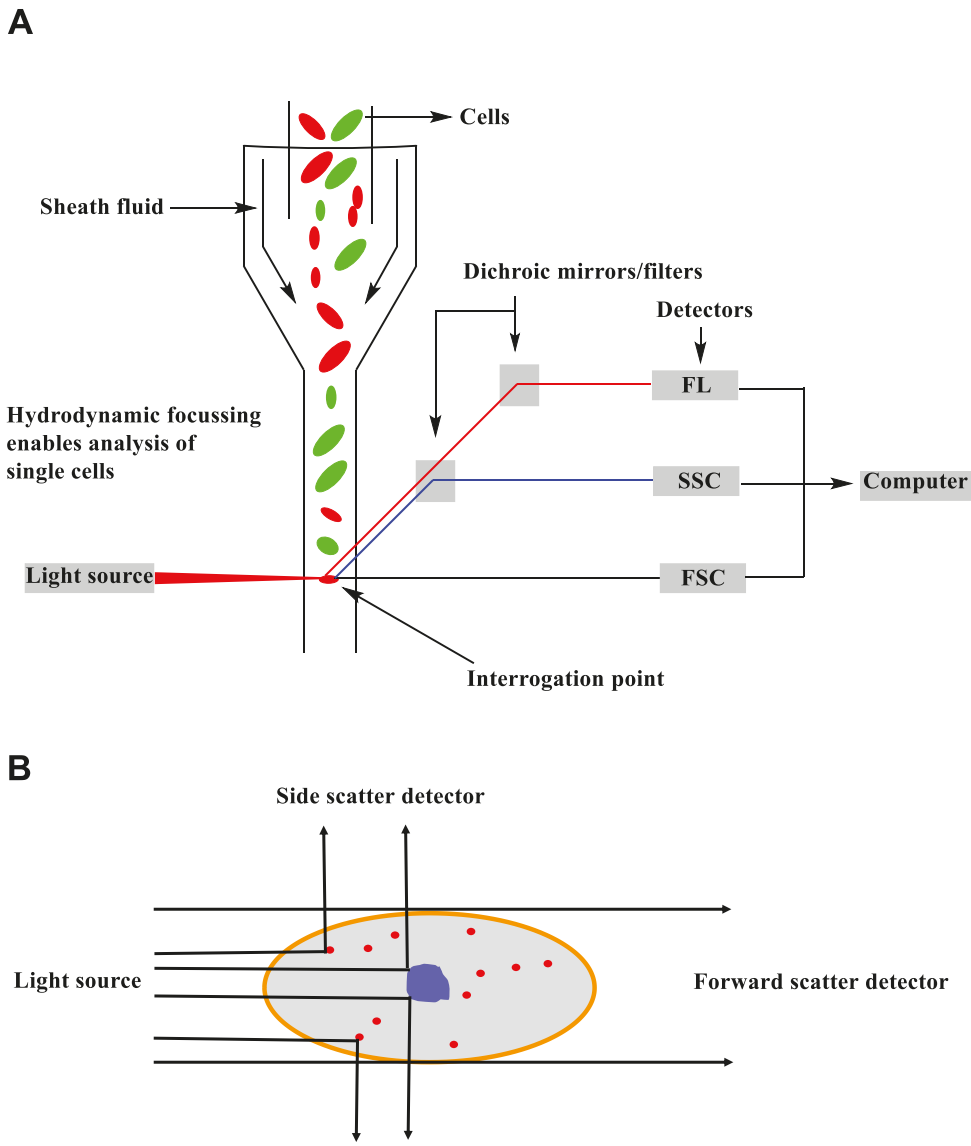


Figure 11: Flow cytometry. (A) Schematic of the flow cytometer instrument. (B) Light scattering profiles after contact with a cell. FSC, forward scatter; SSC, side scatter; FL, fluorescence.

Additional data can be acquired if the cells have autofluorescence or if they are stained using a fluorescent dye detected by the fluorescence (FL) detectors. The light is distributed in the optical system by dichroic mirrors/filters (long pass, short pass, and band pass), which are dependent on the wavelength to the SSC and the fluorescent detectors. This light reaches the detector and is amplified by a

photomultiplier tube (PMT) that converts optical signals into digital signals and which can be represented as dot plots, histograms, or density or contour plots (Díaz et al., 2010; Lencastre Fernandes et al., 2011; Shapiro, 2003).

Before analysing a sample using the FCM, it is important to have standardisation, calibration, control, and validation that provide consistency and reliability in the analysis. It is likewise important to have positive and negative controls as these permit better and more precise interpretation (Vives-Rego et al., 2000; Wang & Hoffman, 2017). The next section deals with fluorescent dyes that have potential applications in LAB.

Fluorescent stains with potential applications for LAB

The application of flow cytometry (FCM) as a tool has revolutionised the study of cellular physiology. Table 5 lists a few of the fluorescent stains that describe the physiology of microorganisms. Applications of FCM include multicolour immunophenotyping (Jaso et al., 2014), intracellular detection of cytokines and transcription factors (Marsman et al., 2020; Yin et al., 2015), analysis of cell cycles (Kim & Sederstrom, 2015; Pozarowski & Darzynkiewicz, 2004), cell enumeration and viability (Collado et al., 2017; Iaconelli et al., 2015; Nescerecka et al., 2016; Yeo et al., 2018), metabolic activity (Kolek et al., 2016; Zotta et al., 2012), microbial strain determination (Buysschaert et al., 2018; Prest et al., 2013), morphology description (Ehgartner et al., 2017; Konokhova et al., 2013; Narayana et al., 2020; Rao et al., 2021), detecting stress tolerances (Bircher et al., 2018; Carlquist et al., 2012; Heins et al., 2019; McBee et al., 2017), and many more.

Fluorescence, a type of photoluminescence, is triggered when a fluorochrome absorbs light, leading to the excitation of an electron from the most occupied orbital to a higher energy state in an unoccupied orbital (Mullins, 2010). The fluorescent stains/dyes must feature the following properties: biologically inert, high extinction coefficient and a high quantum yield (small concentrations of stain can be detected upon activity), narrow emission spectrum to avoid overlapping, photostability, and low toxicity (Díaz et al., 2010).

SYBR Green (SG) or SYTO in combination with propidium iodide (PI) has been extensively used to indicate the viability of a culture (Bensch et al., 2014; Buysschaert et al., 2018; Yeo et al., 2018; Zotta et al., 2012). In Papers I and II, a combination of SG and PI was utilised to give information on viability based on membrane permeability, which led to assigning cells as intact or damaged. The damaged cells displayed two distinct populations, damaged A and damaged B (Paper II), which differ in the amount of SG fluorescence. It is unclear exactly to what degree they differ. These damaged subpopulations have previously been observed in past studies and provides its own way of interpretation (Boda et al., 2015; Léonard et al., 2016; Manoil et al., 2014; Martínez-Abad et al., 2012).

Table 5: Selected fluorescent stains with potential applications for LAB.

Fluorescent dye	Excitation/Emission wavelength	Mechanism of action	Application	References
SYBR-Green (SG)	497/520	SG permeates the membrane and binds to DNA.	Determination of DNA content	Paper I & II, (Bensch et al., 2014; Buysschaert et al., 2018)
Propidium Iodide (PI)	535/617	PI enters cells with compromised membranes and binds to DNA.	Distinguish between intact and damaged cells	Paper I - IV, (Amor et al., 2002; Bunthof et al., 2001; Zotta et al., 2012)
5(6)-carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE)	494/521	CFDA-SE is enzymatically hydrolyzed by cellular esterases into carboxyfluorescein succinimidyl ester, which is fluorescent and hydrophilic, thus making it membrane-impermeant	Distinguish between live and dead cells depending on the esterase activity	Paper III & IV, (Hansen et al., 2016; Rault et al., 2007)
Bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4(3))	490/516	Fluoresces in the microenvironment of a membrane, enters and exits the cell in response to the charge on the plasma membrane	Determine the energy state of the cell	(Bouix & Ghorbal, 2015; Papadimitriou et al., 2006)
CellROX™ green	485/520	Fluorescence upon oxidation by reactive oxygen species (ROS)	Determine ROS levels and oxygen tolerance of the cells	(Manoil, 2018; Zeng et al., 2022)
1,6-Diphenyl-1,3,5-hexatriene (DPH)	350/452	Increase in fluorescence after intercalation into membranes	Detect lipids and determine membrane fluidity	(Li et al., 2011; Velly et al., 2015)

5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) exhibits passive diffusion across cellular membranes. CFDA-SE is initially devoid of colour and fluorescence as its acetate groups remain intact before cleaving. Upon cleavage by intracellular esterases, CFDA-SE generates intensely fluorescent carboxyfluorescein succinimidyl ester (CFSE). The succinimidyl ester moiety of CFSE selectively reacts with intracellular amines, leading in the formation of fluorescent conjugates that exhibit excellent retention within cells (Díaz et al., 2010). The esterase activity correlates with cell activity, and the fluorescence signal

from CFSE is therefore a measure of viability. CFDA-SE can be combined with PI to analyse live, dead, and damaged cells (Hansen et al., 2016). In addition to the esterase activity, the fluorescence spectrum of CFDA-SE correlates with pH and has thus been used to estimate intracellular pH (Breeuwer et al., 1996; Molina-Gutierrez et al., 2002). The probe was found useful for studying the effects of perturbating environmental condition on pH homeostasis. Another application of CFDA-SE that has been studied in LAB is in the analysis of metabolic activity. Cells that are stained with CFDA-SE are treated with different sugars, and only those that are capable of ATP-dependent export lose the fluorescent colour, while cells incubated in buffer without a carbon source remain unchanged (Breeuwer et al., 1996).

Other dyes of interest for studying cells

Bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4[3]) fluoresces when it enters the cell membrane as a result of membrane depolarisation, and it is linked to ATP formation (Bouix & Ghorbal, 2015; Shapiro, 2003). It can also be linked to viability because only live cells are able to maintain membrane potential by regulating ion concentrations across the plasma membrane; however, depolarisation of the membrane potential results in a decrease in cellular activity but not necessarily permanent cell death (Papadimitriou et al., 2006). Due to its lipophilic nature, DiBAC4[3] results in non-specific binding. CellROX™ Green Reagent is a dye for measuring oxidative stress in live cells. It fluoresces upon oxidation by reactive oxygen species (ROS) (McBee et al., 2017). 1,6-Diphenyl-1,3,5-hexatriene (DPH) is a dye used to detect lipids and measure membrane fluidity (Marielle & Sarrah, 2017).

Gating strategies

The process of 'gating' involves the sequential identification and classification of a specific cell population using a panel of molecules that can be visualised by their unique fluorescence emission spectrum. For example, to study the properties of intact cells, one needs to distinguish between intact and damaged cells by creating gates based on the fluorescence dye utilised (Nescerecka et al., 2016). There are multiple ways of performing the gating, but it needs to be validated with positive and negative controls in order to categorise the populations in the right manner (Vives-Rego et al., 2000; Wang & Hoffman, 2017).

Manual gating

A common way to perform gating is by doing it manually, for which computer software (e.g., FlowJo) is utilised. In this approach, gates are user-defined using a graphical interface and are typically set using linear equations. For defined cultures with low amounts of particles/events, the first gate is often based on FSC and SSC because it can be used to separate cells from the background (Figure 12) and can

also separate cells of different sizes (this strategy is often used in microbiology, especially for pure cultures). The result of a gating FSC vs SSC plot is the total cell count, which is later used to gate based on fluorescence detectors. For example, in Figure 12 the cell population is used in plot FL1 (SYBR Green) vs FL3 (propidium iodide) to gate the cells as intact and damaged cells using the computer software FlowJo (Nescerecka et al., 2016; Prest et al., 2013). Damaged cells have higher PI fluorescence as they have a more permeabilised/compromised cell membrane, whereas intact cells are gated based on the negative PI, positive SYBR Green signal.

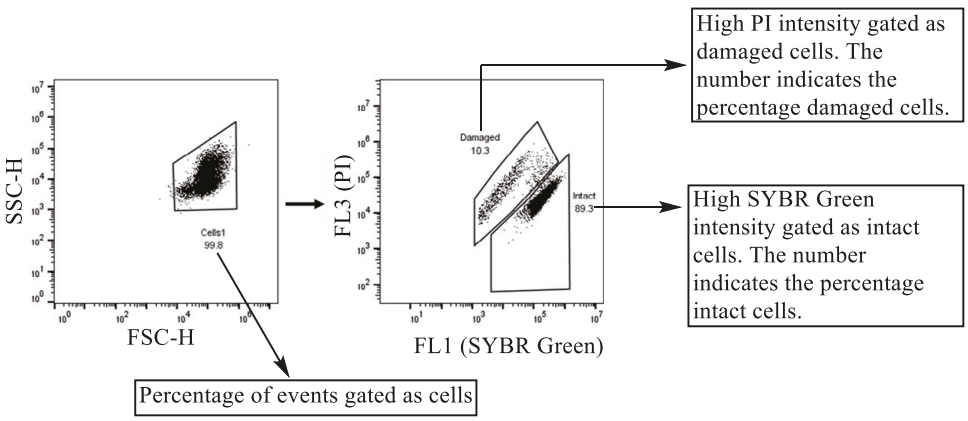


Figure 12: Graphical representation of gating strategy for separating cells from background and subdivision into groups. Scattering profiles (FSC-H vs. SSC-H) were manual gated. A log scale density plot of FL1-H (SYBR Green) vs FL3-H (PI) was obtained to visualize the fluorescence of the dyes that was further sub-grouped in to intact and damaged population.

Manual gating comes with a set of disadvantages:

- There is no universal gating strategy that fits all sample types. As such, it is time-consuming when working with complex gating strategies (Lo et al., 2008; Pyne et al., 2009; Verschoor et al., 2015). Fitting the same gates for all the samples will hamper the outcome as some samples might have a distinct fingerprint. As a result, the true identity of the subpopulations in a sample may not be found as they would be gated as a different subpopulation in another sample.
- There is an user bias associated with manual gating (Ge & Sealfon, 2012; Lencastre Fernandes et al., 2011; Pyne et al., 2009). A subject matter expert is needed to understand the correlation between fluorescence stains used and the subpopulations generated, and there may be a lack of consistency if the same individual performs the gating at two different time points.

Automated gating strategy

The automated gating strategy is a model-based approach to gating the cells. This gating strategy is less labour-intensive, less time-consuming, more dynamic, and less biased in data processing compared to manual FCM workflows. The automated gating strategy may be based on different clustering algorithms to separate the subpopulations. The objective of clustering is to divide a large set of data into smaller, more manageable subsets or clusters that are internally homogeneous and externally heterogeneous. A few of the clustering classes are provided in Table 6.

Table 6: Cluster classification available for gating.

Clustering classification	Concept	Method	Reference
Partition	To consider the centre of the data points as the centre of the cluster to which they belong	*k*-means	(Ge & Sealfon, 2012), Paper II
Hierarchy	Each data point is treated as a separate cluster. The process entails merging the two clusters that are closest in proximity to form a new cluster until only one cluster remains; alternatively, the reverse of this process may also be employed.	SPADE	(Qiu et al., 2011)
Distribution	The distribution-based algorithm clusters data based on proximity from its centre. As the distance from the centre of the distribution increases, the likelihood of a point belonging to the distribution decreases.	Expectation-maximisation algorithm	(Pyne et al., 2009)
Density	Clustering based on density connects high-density regions of examples to form clusters, enabling the clustering of arbitrarily shaped distributions as long as the dense regions can be linked. They do not classify outliers into any cluster.	DBSCAN	(Bakshi et al., 2021)
Grid	Transforming the original data space into a gridded structure with a fixed size to facilitate clustering.	FLOCK	(Qian et al., 2010)

According to Bashashati and Brinkman (2009), there are two learning approaches: (i) supervised and (ii) unsupervised. Supervised methods use previously gated training data sets as a guide to gate new cell populations, whereas unsupervised methods use algorithms to detect patterns in the data with minimal or no user input. An advantage of the unsupervised method is the potential to identify novel patterns or previously unknown cell populations.

In Paper II, a k-means clustering algorithm was applied to separate the subpopulations. Advantages of k-means clustering are that it is simple and easy to implement and fast and scalable (i.e., it can handle large datasets). Moreover, it requires little computer power, enables easy cluster interpretation, and provides consistent and reproducible results.

The k-means clustering algorithm is an unsupervised method in which the number of clusters (k) is defined by the user. In the initial step, k random points are inserted as initial centroids into the data from a random seed. The data is then clustered by assigning each FCM event to the closest centroid. After each iteration, centroids are recalculated. Each event is assigned to minimise the sum of distance to a user-defined number of centroids. After all events have been evaluated, a local minimum of the smallest sum of distance is found and the clustering results are determined by the best sum of distances (Aghaeepour et al., 2011; Ge & Sealfon, 2012). A MATLAB script was developed for clustering FCM data on cultures stained with SYBR Green and PI using the function k-means (Paper II). High correlation was observed between manual gating strategy and automated strategy, indicating that automated strategy can replace the manual strategy. It also saves time in processing hundreds of samples and changes dynamically so that cells can be clustered into different subpopulations, which produces less human intervention and user bias.

Morphology descriptors

Bacteria exhibit a wide variety of shapes and sizes. Though the form of a bacterial species typically remains unaltered for many generations, minor changes can appear in the cell and life cycles, which can be impacted by environmental factors (Van Teeseling et al., 2017). Bacterial shape is primarily dictated by the peptidoglycan (PGL) sacculus, which is present in both gram-positive and gram-negative bacteria. Gram-positive bacteria consist of a 20–80nm-thick PGL layer (Willey et al., 2022). Cell size distributions can serve as an indicator of growth deficiencies and can be used for fermentation process control (Palmfeldt & Hahn-Hägerdal, 2000; Senz et al., 2015; Šušković et al., 2000). Conventionally, cell morphology has been described using light microscope or advanced techniques such as fluorescence microscopy, scanning electron microscopy, tomography, and scanning probe microscopy. Bacterial size has been reported in terms of biovolume, but a disadvantage of this approach is even a small error in measured length and width of cells results in large errors when calculating the volume. Kubitschek (1958) utilised the Coulter counter in tandem with microscopy to determine the dimensions of individual bacteria cells (Kubitschek, 1958), and this was also used as a high-throughput method to determine cell size distribution (Kubitschek, 1969; Kubitschek & Friske, 1986). The principle of the Coulter counter is also used in other techniques for single-cell measurements, such as the flow cytometer. As described earlier, FCM has been used to describe the morphology of cells and has an advantage over the traditional microscopy technique in that it can give information beyond cell size alone. Light scatter signals can provide valuable information on cell morphology (Shapiro, 2003).

In Paper I, cell size from microscopic analysis were determined using length and width of the cell, assuming cells to have a cylinder shape (Biselli et al., 2020;

Konokhova et al., 2013; Trueba et al., 1982) using the software ImageJ 1.52n. Cell size were obtained from the scattering profiles (FSC, SSC and pulse width distributions) of the FCM analysis. The distribution within the sample can be described by the coefficient of variation (CV), robust coefficient of variation (rCV), and skewness using the data obtained from the image analysis as well as FCM data (Amir, 2014; Heins et al., 2019; Koch, 1966; Nakamura & Ishimura, 2010). The CV is a statistical measure of relative variability. It is often used to compare the variability of different datasets, especially when their means are different. A higher CV indicates a higher degree of variability within the dataset. The rCV is a modified version of the standard CV that is less sensitive to outliers or extreme values in a data set. Skewness is the measure of distortion from the normal distribution within a dataset (Figure 13). Normally distributed data will have zero skew. If the skewness value obtained is positive, there is a right skew, whereas if the skewness is negative, there is a left skew. Skewness can give information on the existence of potential subpopulations to the main population (Heins et al., 2019).

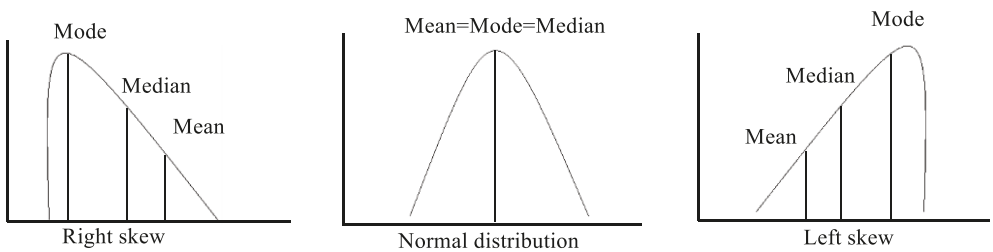


Figure 13: Basic principle of skewness.

Forward and side scattering

FSC gives information on the size of cells/particles and has been experimentally exhibited to correlate with bacterial biovolume (μm^3) (Bouvier et al., 2001; Felip et al., 2007; Foladori et al., 2008). However, I observed no correlation between the FSC and bacterial biovolume as measured by light microscope for *L. reuteri* (Paper I); this can be attributed to the fact that light scatter measurements by FCM are not only determined by cell size. In fact, other properties such as surface roughness and the refractive index difference between the liquid phase and the cells can also contribute to the scattering profiles (Shapiro, 2003). Growth was correlated with the size distribution descriptors (CV, rCV, and skewness). Cultures that achieved high cell densities were composed of less diverse populations of smaller bacteria, while cultures exposed to sub-optimal growth conditions exhibited higher values of CV, rCV, and skewness. As noted, exposure to sub-optimal conditions for cellular growth and function can elicit a stress response in the cell, which may then lead to alterations in its morphological characteristics (Paper I).

Pulse width

Pulse width is another potential morphology descriptor that has not been explored in depth. Pulse width correlates to the time it takes for a cell or particle to pass the laser interrogation point and provides information on cell size and shape. For example, larger cells or particles will generate a broader pulse width signal than smaller cells or particles. Similarly, irregularly shaped cells or particles will generate a wider pulse width signal than cells or particles with a more regular shape (Chioccioli et al., 2014; Hoffman, 2009; Sharpless & Melamed, 1976) (Figure 14). Pulse width has been used previously to distinguish doublets from singlets during cell cycle analysis (Kang et al., 2010; Rico et al., 2022; Wersto et al., 2001).

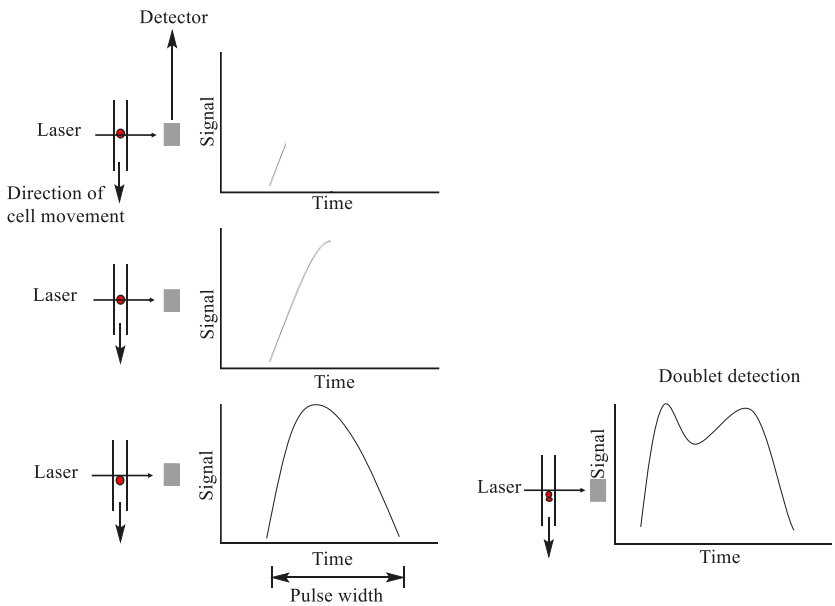


Figure 14: Pulse width parameter used to distinguish between singlet and doublets.

The pulse width distribution can be visualised on a FCM histogram, where the x-axis represents pulse width and the y-axis represents the number of events (i.e., cells or particles) with a given pulse width (Figure 15). By analysing the shape and characteristics of the pulse width distribution, insight can be gained into the heterogeneity of the cell population being studied (Paper I). For example, in a mixture of cells of different sizes, a multimodal pulse width distribution may indicate the presence of many distinct subpopulations.

Pulse width distributions were studied by gating subpopulations based on minimum peaks (Figure 15). The initial gate is characterized by cells of smaller length, with a proportional increase in cell length leading to the cells being sorted into successive

gates. Damaged cells always have lower pulse width values than intact cells (Paper I). To the best of my knowledge, there has been no reported use of pulse width as a marker of cell morphology in process analytical technology (PAT) for fermentation processes.

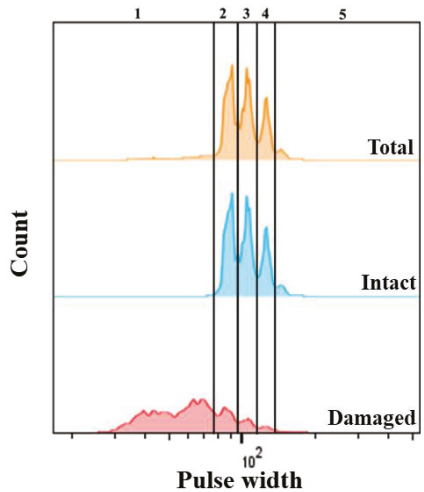


Figure 15: Gating on pulse width distribution to study population heterogeneity.

Online flow cytometry

Online FCM is particularly useful in applications where rapid and accurate analysis of large numbers of cells or particles is required, such as in environmental monitoring or process control in the biopharmaceutical industry. It has previously been used to trace bacterial growth in membrane systems (Mimoso et al., 2015), drinking water analysis (Besmer et al., 2014; Hammes et al., 2012), community tracking of lactobacilli (Buysschaert et al., 2018), and monitoring of algae (Haberkorn et al., 2021).

Online FCM is an automated system which draws samples from the source (e.g., bioreactor), dilutes the sample in buffer, stains it with a fluorescent dye, incubates it, and sends it to the FCM for analysis. There are both advantages and disadvantages with this system. The advantages of this setup are as follows: (i) it performs real-time analysis that is useful for monitoring rapidly changing cellular events; (ii) automated analysis and less sample handling reduces the need for human intervention; this, in turn, (iii) reduces the risk of contamination and improves the accuracy of the results. A few of the disadvantages with this system include fluctuations in data quality due to it being sensitive to the accumulation of debris from previous samples during continuous monitoring. It can also have limited

sample compatibility as it might not be useful for complex or specialised samples when filtering of larger particles is required.

Utilising this system allows one to complement the traditional way of measuring growth by obtaining even more information regarding cell physiology, such as morphology and particular cell function (based on the fluorescent dyes used). Online FCM coupled with automated gating strategy makes the FCM analysis easier to perform (Paper II).

4. Impact of process parameters on freeze-drying stress tolerance of probiotic products

Process parameters during production of probiotics can influence the survival and growth of lactic acid bacteria (LAB). The ability of LAB cells to tolerate process conditions is a complex characteristic that relies on various intrinsic and extrinsic factors. Extrinsic factors can be managed by employing PAT tools and process regulation. In contrast, intrinsic factors are determined by genetic code, gene expression regulation, and cell metabolism, which are in a state of dynamic equilibrium with the environment and which depend on cellular responses. Therefore, the optimal process conditions are strain-specific and must be adjusted for each strain. The combination of strain and process engineering in an iterative design-build-test cycle is the optimal approach to maximising product quality, as defined by the highest viability, stability, and probiotic activity. However, the molecular mechanism of specific beneficial cell properties that underlie maximal product quality is in many ways unknown, posing a significant challenge.

For the final product to fulfil probiotic product specifications (high viability & stability), the cells need to possess a number of specific properties. FD process robustness and surviving long-term storage are two key properties. The prerequisite for tolerating FD stress and subsequent administration is firstly determined by the fermentation step, which aims to generate the fittest and most tolerant cells. Engineering of the subsequent process steps is more aimed at minimising the environmental pressure put on the cells either by formulation or controlled rehydration.

The principle underlying freeze-drying

FD, also known as lyophilisation, is a process used to remove water from a substance while preserving its physical and chemical properties. The principle behind FD is sublimation, whereby water molecules are converted directly from a solid (ice) by freezing to a gas (water vapour) and subjecting it to a vacuum without

passing through a liquid state. This process is commonly used in the pharmaceutical, food, and biotech industries to stabilise sensitive materials such as proteins, enzymes, and microorganisms. FD is done both to preserve biological materials for long-term storage and transportation as well as to create a dry, stable, and potent product that can be easily rehydrated when needed. FD of products is an ideal choice for probiotic manufacturers and distributors as they are lightweight and easy to transport and store

The following are the general steps involved in FD:

1. Pre-formulation: The material to be freeze-dried is typically prepared by adding a lyophilisation protectant (lyoprotectant; e.g., sucrose) to protect the material from damage during the FD process.
2. Freezing: The material is frozen to a temperature well below its freezing point, typically between -40 °C and -80 °C.
3. Primary drying: The frozen material is placed in a vacuum chamber, and the pressure is lowered to a level where the frozen water molecules can sublime directly from the solid state to a vapor state without passing through the liquid state. This process is called sublimation, and it is the primary means by which water is removed from the material.
4. Secondary drying: After the majority of the water has been removed by sublimation, any remaining moisture is removed via a process called desorption. The temperature is raised slightly above the freezing point, and the remaining water molecules are removed by evaporation.

The duration of the process depends on the material being dried, the size of the sample, and the specific conditions used. The process can take anywhere from several hours to several days. Before FD, cells are usually treated with a lyoprotectant to protect them from damage during the drying process. Lyoprotectants are typically small molecules that can stabilise cellular structures and prevent damage caused by ice crystals. Examples of lyoprotectants include sugars (such as trehalose and sucrose), polyols (such as mannitol and sorbitol), and amino acids (such as glycine and alanine). FD is different from spray drying, which uses high temperatures to evaporate water from a liquid to yield a dried powder that may not preserve the structure and activity of the biological material. The suitability of FD as a preservation (long-term storage) method may depend on the specific characteristics of the bacterial strain, such as its resistance to low temperatures and the presence of protective compounds that can prevent damage to the bacterial cells during the drying process. The market for freeze-dried probiotic products is well-established and growing, driven by consumer demand for functional foods and supplements.

Process parameters and cell responses

Cells experience several transitions during environment changes throughout the steps involved in the preparation of FD cultures (fermentation, pre-formulation, FD, and rehydration). In this changing environment, cells are adapting their metabolic and structural networks for optimal performance in the unfamiliar environment. The different environmental shifts pose different stresses on the cells, and cells can be more or less pre-equipped to manage those changes. There are different strategies to improve their survivability. In general, subtle and/or slow gradual changes are better for the cells to cope with transitions. Thus, the previous environment that cells have experienced sets the cells' ability to survive in the succeeding environment to a significant degree. In this section, the focus is on how cells respond to process parameters during cultivation (Figure 16; Table 7).

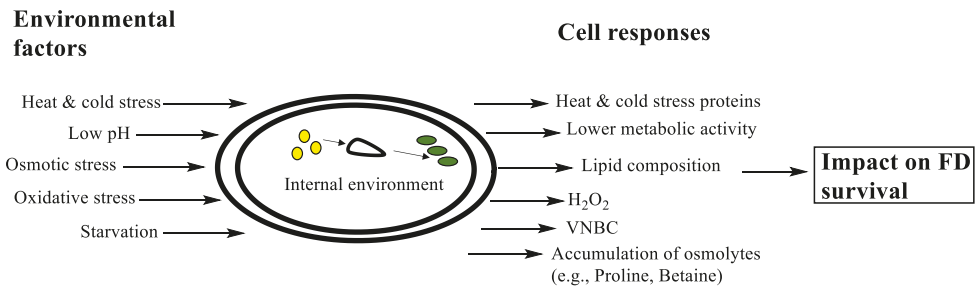


Figure 16: Cell responses to environmental factors.

Temperature

Fermentation temperature can influence the stress tolerance of LAB during FD. The temperature at which the bacteria are grown prior to FD can affect their physiology, including their membrane composition, metabolic activity, and stress response mechanisms. Sub-optimal temperatures (low and high) have been shown to have a positive impact on freezing and FD. At high temperatures cell membranes become more fluid, whereas at low temperatures cell membranes become more rigid and less flexible; this has been mainly attributed to the increase in either cyclopropane-ringed FA or an increase in UFA that leads to higher membrane fluidity (Fernández Murga et al., 2000; Liu et al., 2014; Lorca & de Valdez, 1999; Schoug et al., 2008; Wang et al., 2005).

Heat shock proteins (HSPs) and cold shock proteins (CSPs) are produced as a result of heat and cold stress, respectively. HSPs promote the correct folding of growing polypeptides, assembly of protein complexes, degradation of damaged proteins which cannot be properly refolded, and translocation of proteins (De Angelis &

Gobbetti, 2004; Papadimitriou et al., 2016). CSPs increase membrane fluidity in the lipids (by disrupting the interactions between the lipids in the membrane), efficiency of transcription (CspB has been shown to promote DNA melting, which can help to facilitate the binding of transcription factors and RNA polymerase to the DNA), and translation (CspD protein interacts with RNA polymerase and promote transcription at low temperatures) so that cells are adapted to survive in those conditions (De Angelis & Gobbetti, 2004; Keto-Timonen et al., 2016).

pH

Probiotic bacteria experience a drastic change in environmental conditions when they encounter gastric acids in the stomach (pH 2) and the intracellular acidification of lactic acid. However, the ability to adapt to low pH levels, which is commonly referred to as the acid-tolerance response, has a beneficial impact on surviving in acidic environments, though there is no positive impact of this on FD (Hernández et al., 2019; Schoug et al., 2008).

Few mechanisms that LAB adopt to acid stress are stated. LAB adapts by modifying membrane fluidity through modulating fatty acid composition (Liu et al., 2014; Yang et al., 2015); producing acid stress proteins such as DnaK, GroEL, and GroES (Fernandez et al., 2008; Wu et al., 2014); and promoting expression levels of proton pumps (Koponen et al., 2012; Sánchez et al., 2007) to counteract intracellular acidic pH. LAB also upregulates proteins associated with glycolysis to optimise its carbohydrate energy sources (Wu et al., 2012; Zhai et al., 2014), consumes excessive cytoplasmic protons which can be utilised for the decarboxylation of glutamate, and generates alkaline products (e.g., gamma-aminobutyric acid [GABA]) to increase the cytoplasmic pH (Feehily & Karatzas, 2013; Teixeira et al., 2014).

Oxygen

Oxygen can have both good and harmful effects on bacteria. Oxygen is one of the most significant electron acceptors for the recycling of reduced co-factors (Gänzle, 2015). As discussed in Chapter 2, NAD+ is regenerated by NADH oxidase, which directs the flux towards formation of acetate via the generation of additional ATP. At the same time, O_2 can give rise to the formation of reactive oxygen species (ROS) that react and cause damage to proteins, lipids, and nucleic acids (Papadimitriou et al., 2016). Higher UFAs in the presence of air were observed, coinciding with high cell survival after FD stress (Paper II). In contrast, anaerobic cultivations effected negatively on FD survival (Papers II, III, and IV).

Exogenous fatty acid

Tween 80, a type of surfactant, has been demonstrated to enhance the proliferation of bacteria in growth media as well as to increase their resistance to post-harvest treatments, such as survival in highly acidic conditions and long-term storage (Corcoran et al., 2007; Hansen et al., 2015; Johnsson et al., 1995; Li et al., 2011; Partanen et al., 2001; Tan et al., 2012; Terraf et al., 2012). This outcome is attributed to the presence of oleic acid that has been incorporated into the cell membrane, which increases the concentration of UFAs. However, I did not observe any improvement in FD survivability with the presence of Tween 80 in the medium (Paper III).

Pre-formulation hold time

Pre-formulation hold time (PHT) is the time between the pre-formulation step and freezing. The duration of PHT has not been thoroughly examined as a parameter in the fermentation process as it often falls outside the scope of research during strain development and scaling. The term PHT describes a process of pre-formulation in which time is one of the variables. The sucrose content, sucrose conversion, metabolite formation (e.g., fructose, exopolysaccharides, etc.), and influence of pH on long-term stability were studied (Paper IV). Neglecting to investigate this aspect may lead to process variability, potentially impacting the effectiveness of the resulting product (Fenster et al., 2019). In Paper IV, bacteria were subjected to three conditions, namely (i) being directly frozen, (ii) 3h PHT, and (iii) 3h PHT + additional lyoprotectant based on how much lyoprotectant was converted during the 3h PHT. There was metabolic activity during the PHT, and sucrose was converted primarily to fructose, possibly exopolysaccharides (EPSs) and few organic acids which led to decrease in environment pH. An accelerated shelf-life study displayed that direct freezing had better stability compared to the other two conditions. Similar results were observed for various LAB when they were let to convert cryoprotectant during freezing and FD (Cui et al., 2018).

Table 7: Cell response to process parameters during cultivation.

Factor	LAB strain	Process parameter	Cell response	Improvement	References
Temperature	*L. acidophilus* CRL 640	Sub-optimal temperature (25 °C)	Poor growth, increase in C18:2 and C16:0 FA	More resistance to the freeze–thaw process	(Fernández Murga et al., 2000)
	L. reuteri I5007	47 °C during stationary (Short adaptation step)	Lowered growth rate, increased UFA:SFA	Improved survival following FD	(Liu et al., 2014)
	L. coryniformis Si3	Cultivation at 42 °C (Short adaptation step)	Lowered growth rate, high content of UFA	Improved survival following FD	(Schoug et al., 2008)
	L. acidophilus CRL 639	Cultivated at sub-optimal temperature (25 °C)	Poor growth, morphology change from short rods to long filaments, cold shock proteins activation	Contribute to cold acclimation by maintaining some metabolic functions of the cells	(Lorca & De Valdez, 1999)
	L. reuteri DSM 17938	Cultivation at 30 °C, 37 °C, 44 °C	Lowered growth, long chains oberved at 30 °C	Improved survival following FD for 30 °C and 37 °C, poor survival for 44 °C	Paper I
pH	*L. reuteri* ATCC 55730	Cultivation at pH 5 and 6 and harvest after reaching 0.5, 2.5 and 4.5h after reaching stationary	pH 6 grown cells exhibited elongated morphology but a slightly higher gowth rate compared to pH 5	Improved survival following FD when the cells were grown at pH 5 and harvested after 2.5 h in the stationary phase	(Palmfeldt & Hahn-Hägerdal, 2000)
	L. reuteri I5007	Neutral pH during stationary (Short adaptation step)	Increased UFA:SFA	Improved survival following FD	(Liu et al., 2014)
	L. reuteri DSM 17938	Low pH 4.5	No significant difference in growth rate	Poor FD survival but improved resistance to low pH and bile salts	(Hernández et al., 2019)
	L. coryniformis Si3	Cultivation pH 4.5	No significant difference in growth rate	Poor FD survival	(Schoug et al., 2008)
	L. acidophilus	Cultivation at pH 5	High cycC19:0 content	Increased resistance to freezing	(Wang et al., 2005)
	L. casei	Cultivation at pH 5	Increase in Intracellular pH, increase in H+-ATPase activity, increase in intracellular ATP pool	Improved survival at pH 3.3	(Wu et al., 2012)

Factor	LAB strain	Process parameter	Cell response	Improvement	References
Oxygen	*L. reuteri* DSM 17938	Low levels of oxygen	No significant difference in growth rate, but metabolic shift observed, increased UFA:SFA	Improved survival following FD	Paper II
Exogeneous FA	*L. reuteri* DSM 17938	Addition of Tween 80	Growth enhancement, low UFA	Poor FD survival similar to medium without Tween 80	Paper III
Pre-formulation hold time	*L. reuteri* DSM 17938	Direct freeze	Normal growth	High survival rate during storage	Paper IV
	Various LAB	Direct freeze	Damage to ATPase, b-galactosidase, and cell membrane fluidity due to acid production from the conversion of cryoprotectant	Poor FD survival	(Cui et al., 2018)
Osmotic stress	*L. buchneri* R1102	Addition of KCl	Betaine accumulation	High survival rate during storage	(Louesdon et al., 2014)
	L. Salivarius	0.8 M NaCl in media	Proline accumulation	NR	(Qi et al., 2018)
	L. delbrueckii subsp. bulgaricus	0.2 M NaCl in media	Glycine betaine accumulation	Increased resistance against osmotic stress	(Han et al., 2018)
Starvation	*L. rhamnosus* GG	Cells harvested at late stationary phase	Higher glycolytic in pH-controlled fermentation	FD survival improved in pH uncontrolled fermentation	(Ampatzoglou et al., 2010)
Other factors	*Lactobacillus reuteri* TMW1.106	Cryoprotectant- fructo-oligosacchardies	Direct interaction with membrane led to increased membrane fluidity	Improved survival following FD	(Schwab et al., 2007)

Osmotic stress

Osmotic stress is a physiological response that occurs in cells when they are exposed to a solution with a different concentration of solutes (such as salts or sugars) than the internal environment of the cell. Exposure to hyperosmotic stress triggers the movement of water out of the cell, resulting in decreased cell turgor pressure, altered intracellular solute concentrations, and a change in cell volume. This decrease in turgor pressure is associated with decreased growth rates, survival rates, and metabolic activity. To combat this, lactobacilli possess solute accumulation mechanisms that allow them to retain water within the cell and thus to maintain turgor pressure (De Angelis & Gobbetti, 2004; Papadimitriou et al., 2016; Van De Guchte et al., 2002). In the presence of KCl and NaCl in media, LAB could overcome hyperosmotic stress by accumulating glycine-betaine, betaine, and proline (Han et al., 2018; Louesdon et al., 2014; Qi et al., 2018).

Starvation

Nutrient starvation results in cells entering the stationary phase, where the cellular mode of operation is changed from the maximal growth rate to a dormant state, with a global rearrangement of material and activities to promote long-term survival. This implies a reduction in metabolic activity to a resting state adapted to maintenance and restricted use of stored energy in the form of storage carbohydrates, as well as recycling of proteins and other constituents. There are several types of starvation. For example, carbohydrate starvation leads to cell energy depletion, phosphate starvation is unfavourable for the energy supply and DNA/RNA synthesis, and nitrogen starvation results primarily in the limitation of protein synthesis.

Experimental evidence suggests that under conditions such as stationary-phase growth, biofilm formation, starvation, and other stress-inducing scenarios, certain bacteria enter a viable but non-culturable (VBNC) state. In this physiological state, the cells retain metabolic activity and acquire resistance to multiple stresses, yet they are incapable of proliferation (Papadimitriou et al., 2016). This is the reason cultures are allowed to reach stationary phase and remain there for some time before harvesting (Paper II). Cells in the exponential phase are more sensitive to FD stress (Hua et al., 2009).

5. Conclusions

The FD survival of microbial cultures depends on numerous parameters. I studied the impact of process variables on the FD tolerance of *Limosilactobacillus reuteri* DSM 17938. My main conclusions are presented below.

- *L. reuteri* DSM 17938 can tolerate low levels of oxygen in a well-controlled bioreactor setup. Oxygen had no impact on growth rate, but as it is an electron acceptor, there was modulation of redox metabolism, and acetate formation was observed along with lactate formation. (Paper II)
- Increased membrane fluidity (UFA:SFA) was observed when *L. reuteri* DSM 17938 was cultivated in the presence of oxygen, which may explain the high FD survivability. Conversely, nitrogen sparging cultivations displayed poor FD survival. This could be due to a lower UFA concentration in the cell. (Paper II)
- A FCM pipeline for evaluating the relationship between environmental parameters and cell morphology of *L. reuteri* DSM 17938 during cultivation was developed. High robustness of *L. reuteri* DSM 17938 towards FD stress coincided with good growth and a cell population consisting of smaller cells and a narrow size distribution. Change in temperature induced the highest change in morphology, whereas oxygen had the highest impact on cell growth. (Paper I)
- Pulse width was successfully applied for *L. reuteri* DSM 17938 cells. This tool is one of the morphology descriptors that was used to describe morphology heterogeneity, which hitherto had not been used as a marker of cell morphology in PAT for fermentation processes. The advantage of pulse width is in its capacity to distinguish smaller cells from larger ones. (Paper I)
- A FCM pipeline for automated sampling and gating was developed for analysing the cell count, viability, and morphology of the cells to minimise human intervention and quicken data processing. (Paper II)
- A defined mineral medium (DMM1) with 14 essential amino acids required for the growth of *L. reuteri* was developed to study the effect of an exogeneous FA source. DMM1 can be useful for studying the metabolic pathways of the microorganism. (Paper III)

- Addition of Tween 80 to the media improved the biomass yield and growth rate and shortened the cultivation time. (Paper III)
- The medium without Tween 80 had production of mostly SFAs, including palmitic and stearic acid, which suggests that *L. reuteri* DSM 17938 may not be able to *de novo* synthesise UFA efficiently without an exogeneous FA source. (Paper III)
- Pre-formulation hold time (PHT) resulted in sucrose (lyoprotectant) conversion, but the carbon recovery was low; this could be attributed to EPS production. (Paper IV)
- The accelerated shelf-life study indicated that samples that were directly frozen were more stable over time compared to samples with 3 h PHT and 3 h PHT with additional sucrose. (Paper IV)

6. Outlook

Process parameters have a significant impact on cell physiology and determine tolerance to FD and subsequent stress (acid and bile). The knowledge gained from my studies improves our understanding of the impact of process parameters during the fermentation of probiotics production. At the same time, the findings have given rise to new research questions.

- Many research articles, including Paper II, have determined that high membrane fluidity correlates with high FD stress tolerance. There was no indication of *L. reuteri* having a fatty acid desaturase based on current gene annotations and the protein domain analysis (Paper II). However, there is still uncertainty over how *L. reuteri* produces its UFAs. There is a need to further investigate the synthesis of lipids and how this correlate to FD tolerance.
- Nitrogen sparging is widely used in the research field to maintain the anaerobic production of probiotics. I observed that anaerobic cultivations resulted in lower survival after FD (Paper II, III & IV) when compared to air-sparged conditions. This finding leads to the question of how oxygen benefits the cells, and whether it acts as a prestress condition, helping to overcome the stress of FD. Performing transcriptomics or proteomics can help us gain a better understanding of the effects of air/anaerobic conditions on the cells.
- Scaling up is one of the most challenging aspects of industrial processing. Increased pre-formulation hold time was observed to negatively impact the shelf-life of the probiotic product, as seen in Paper IV. Other challenges in large-scale fermentation processes compared to laboratory-scale fermentations are (i) maintaining the same degree of strict regulation of environmental conditions and (ii) greater difficulty in achieving homogeneity.
- All four studies used the lyoprotectant sucrose and kept the FD program constant. It would be interesting to study more complex formulations as this would increase understanding of the stability of bacteria over extended periods of time. Similarly, employing different FD programs allows control over the structure of the matrix, which in turn informs comprehension of the long-term stability of probiotic bacteria.

- Fast and informative monitoring of cell physiology is important. Dyes for assessing membrane fluidity using FCM aid in understanding subsequent FD stress tolerance. Ongoing improvements to automated gating strategies for FCM are making it easier and faster to analyse large and complex datasets while also improving the accuracy, reproducibility, and objectivity of the analysis. Pulse width distribution can be used to replace PI staining, as majority of damaged cells end up in pulse width gate 1 which suggests that PI^+ cells may have unique pulse width fingerprint. Although this needs further investigation. FCM counts will compliment if not replace the heterotrophic plate count (HPC) as FCM provides a quick analysis. FCM can give more information than HPC in terms of cells' VBNC state cells status, morphology, and other cell properties such as lipid metabolism using DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) and measuring bacterial adhesion to intestinal cells using fluorescein isothiocyanate (FITC)-conjugated dextran as shown previously (Dean et al., 2020; Tsai et al., 2023). The implementation of new monitoring techniques will allow for a deeper understanding of cellular properties, which will be essential to gaining the ability to control and predict FD tolerance and thus in the future provide a probiotic product that has better shelf life stability.

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