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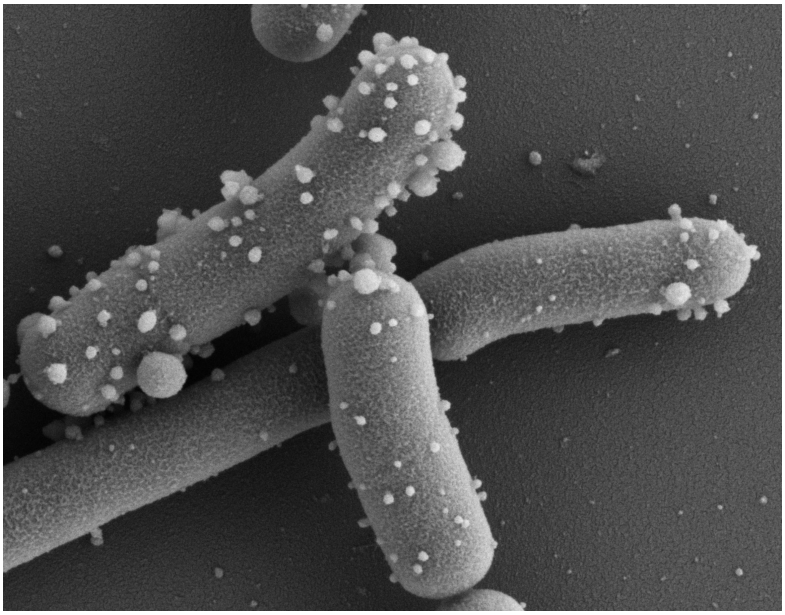
An exploratory journey into probiotic interactions

- Bioactive properties of *Limosilactobacillus reuteri* and *Bifidobacterium longum*

Ludwig Ermann Lundberg



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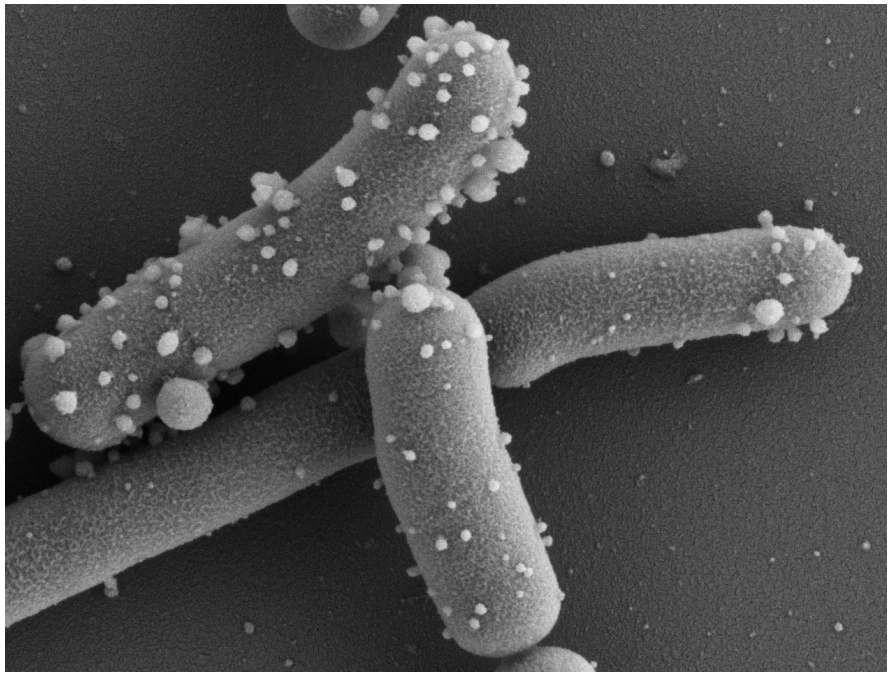

Cover: *Limosilactobacillus reuteri* DSM 17938 with membrane vesicles budding from the cells. (Photo: Anna Pielach, Centre for Cellular Imaging, Core Facilities, Gothenburg University)

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An exploratory journey into probiotic interactions

Abstract

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The mechanisms by which probiotics affect the host are based on one of two principles: *i*) directly, by interacting with specific targets, or *ii*) indirectly, by contributing to the ecological niche constructions of the microbiota. This thesis examined how probiotic bacteria interact with each other and with the host, and how production of probiotics can be manipulated to increase biological functionality, ultimately benefiting the host.

Limosilactobacillus reuteri DSM 17938 is a well-documented probiotic strain, but the mechanisms by which it alleviates infantile colic, combats inflammation, and interacts with the immune system are not well understood. However, several features related to the bioactive properties of DSM 17938 may partly explain these interactions. The features in question are extracellular membrane vesicles (MV), exopolysaccharides (EPS), enzymes, and other metabolites. MV and EPS were evaluated in different models of host interactions, aimed at reflecting possible mechanisms of action in infantile colic. Multifunctionality among the MV was demonstrated and it was also shown that the amount and activity of bioactive components can be altered by optimizing production parameters. Further, a novel strain of *Bifidobacterium longum* subsp. *longum* was described and shown to be a potent fiber degrader, able to stimulate growth and bioactivity of DSM 17938 and its MV *in vitro*. Bifidobacteria and lactobacilli are important in ecological niches of the human gut and the interactions between these bacteria may be key to understanding how to fight inflammatory diseases and disorders using probiotics.

Keywords: *Limosilactobacillus reuteri*, extracellular membrane vesicles, bioactive components, *Bifidobacterium longum,* lyoconversion

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En upptäcktsfärd mot probiotiska interaktioner

Sammanfattning

Probiotika definieras som levande mikroorganismer som när de administreras i tillräckliga mängder ger en hälsofördel för värden. Probiotika kan påverka värden på två principiellt olika sätt, antingen via direkta interaktioner med receptorer och måltavlor eller via deltagande i konstruktionen av ekologiska nischer i tarmens mikrobiota. Avhandlingen syftade till att förstå hur probiotiska bakterier kan interagera med varandra och med människovärden såväl som att förstå huruvida bioaktiviteten hos de probiotiska bakterierna och dess metaboliter påverkas genom att variera produktionsstrategin. *Limosilactobacillus reuteri* DSM 17938 är en av världens mest studerade probiotiska stammar i kliniska studier, men mycket återstår att kartlägga, såsom hur DSM 17938 lindrar spädbarnskolik, bekämpar inflammation och interagerar med immunförsvaret. Det skulle delvis kunna förklaras av de bioaktiva komponenter som DSM 17938 producerar, såsom extracellulära membranvesiklar (MV), exopolysackarider (EPS) och bioaktiva enzymer. MV och EPS utvärderades i flera värdcellsinteraktionsmodeller som syftade till att reflektera förmodade mekanismer inom spädbarnskolik. Resultaten visade att MV är multifunktionella, och att mängd och funktion av bioaktiva strukturer påverkas av produktionsstrategin. En ny stam av *Bifidobacterium longum* subsp. *longum* beskrivs också. Stammen har starka fibernedbrytande egenskaper och kan stimulera tillväxt och bioaktivitet hos DSM 17938 samt dess MV. Bifidobakterier och laktobaciller är viktiga spelare i tarmekologin och genom att förstå interaktioner mellan dessa kan vi potentiellt förstå hur probiotika kan lindra inflammatoriska sjukdomar och åkommor.

Nyckelord: *Limosilactobacillus reuteri*, extracellulära membranvesiklar, bioaktiva komponenter, *Bifidobacterium longum,* lyokonvertering

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I have found that it is the small things, everyday deeds of ordinary folk, that keeps the darkness at bay.

Gandalf (J. R. R. Tolkien ~ The Hobbit)

J. R. R. Tolkien

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Yanhong Pang*, **Ludwig Ermann Lundberg***, Manuel Mata Forsberg, David Ahl, Helena Bysell, Anton Pallin, Eva Sverremark-Ekström, Roger Karlsson, Hans Jonsson & Stefan Roos (2022). Extracellular membrane vesicles from *Limosilactobacillus reuteri* strengthen the intestinal epithelial integrity, modulate cytokine responses and antagonize activation of TRPV1. *Frontiers in Microbiology*, 13:1032202. DOI: 10.3389/fmicb.2022.1032202

II. **Ludwig Ermann Lundberg**, Punya Pallabi Mishra, Peidi Liu, Manuel Mata Forsberg, Eva Sverremark-Ekström, Gianfranco Grompone, Sebastian Håkansson, Caroline Linninge & Stefan Roos. *Bifidobacterium longum* strains boost *Limosilactobacillus reuteri* DSM 17938 and its extracellular membrane vesicles (manuscript).

III. **Ludwig Ermann Lundberg**, Manuel Mata Forsberg, James Lemanczyk, Eva Sverremark-Ekström, Corine Sandström, Stefan Roos & Sebastian Håkansson. Lyoconversion: *Limosilactobacillus reuteri* produce bioactive components during pre-formulation in sucrose (manuscript).

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* Indicates shared first authorship

The contribution of Ludwig Ermann Lundberg to the papers included in this thesis was as follows:

I. Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, and writing - initial draft, review and editing (shared first authorship with YP).

II. Conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, validation, visualization, and writing - original draft, review & editing.

III. Conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, validation, visualization, and writing - original draft, review & editing.

During the timeframe of this thesis work, Ludwig Ermann Lundberg also contributed to the following scientific output:

Rao, N.S., **Ermann Lundberg, L.**, Tomasson, J., Tullberg, C., Brink, D.P., Palmkron, S.B., van Niel, E.W.J., Håkansson, S., & Carlquist. M. (2023). Non-inhibitory levels of oxygen during cultivation increase freeze-drying stress tolerance in *Limosilactobacillus reuteri* DSM 17938. *Frontiers in Microbiology 14:1152389.*
https://doi.org/10.3389/fmicb.2023.1152389

Rao, N.S., **Ermann Lundberg, L**., Palmkron, S., Håkansson S., Bergenståhl, B., & Carlquist, M. (2021). Flow cytometric analysis reveals culture condition dependent variations in phenotypic heterogeneity of *Limosilactobacillus reuteri*. *Scientific Reports 11, 23567.* https://doi.org/10.1038/s41598-021-02919-3

Berglund, B., Hoang, N.T.B., **Lundberg, L**., Kien Le, N., Tärnberg, M., Nilsson, M., Bornefall, E., Thi Khanh Khu, D., Welander, J., Thanh Le, H., Olson, L., Minh Dien, T., E. Nilsson, L., Larsson, M., & Hanberger, H. (2021). Clonal spread of carbapenem-resistant *Klebsiella pneumoniae* among patients at admission and discharge at a Vietnamese neonatal intensive care unit. *Antimicrobial Resistance & Infection Control 10, 162.* https://doi.org/10.1186/s13756-021-01033-3

Sriram, K.K., Ekedahl, E., Hoang, N.T.B., Sewunet, T., Berglund, B., **Lundberg, L**., Nematzadeh, S., Nilsson, M., E. Nilsson, L., Kien Le, N., Minh Tran, D., Hanberger, H., Olson, L. Larsson, M., Giske, C., & Westerlund, F. (2021). High diversity of bla_{NDM-1}-encoding plasmids in *Klebsiella pneumoniae* isolated from neonates in a Vietnamese hospital. *International Journal of Antimicrobial Agents, 106496, ISSN 0924-8579. https://doi.org/10.1016/j.ijantimicag.2021.106496.*

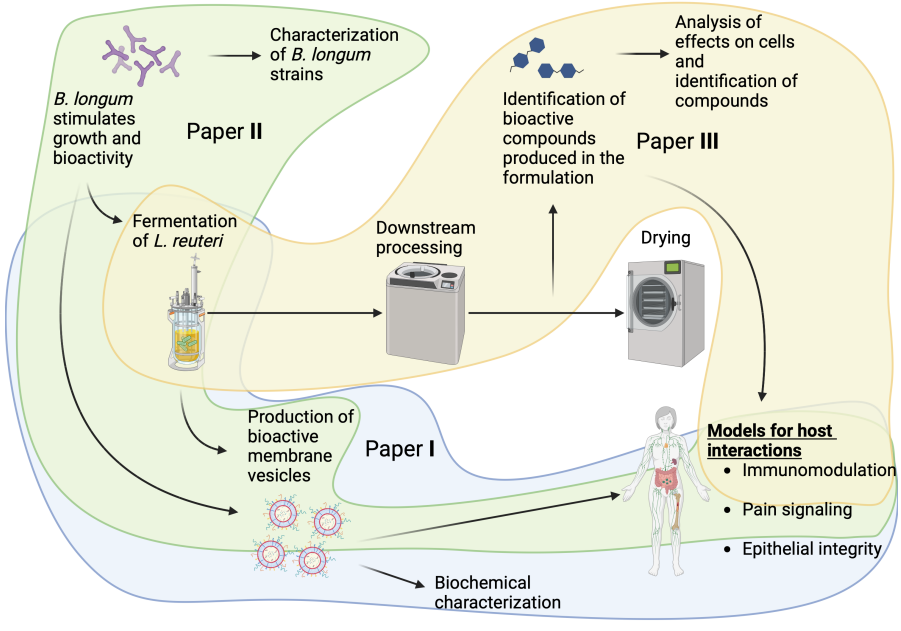
Forsberg, M., Björkander, S., Pang, Y., **Lundqvist, L**., Ndi, M., Ott, M., Buesa Escribá, I., Jaeger, M-C., Roos, S., & Sverremark-Ekström, E. (2019). Extracellular membrane vesicles from lactobacilli dampen IFN-γ responses in a monocyte-dependent manner. *Scientific Reports 9, 17109.* https://doi.org/10.1038/s41598-019-53576-6

Abbreviations

5'NT	5' nucleotidase
AhR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
B. longum	*Bifidobacterium longum*
CAGR	Compound annual growth rate
CFU	Colony-forming units
EPS	Exopolysaccharides
ETEC	Enterotoxigenic Escherichia coli
EU	European Union
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
GCMS	Gas chromatography mass spectrometry
GH	Glycoside hydrolase
GMO	Genetically modified organism
GPCR	G-protein coupled receptor
HCA_3	Hydroxycarboxylic acid receptor 3
HMO	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
IgA	Immunoglobulin A

IL-1β	Interleukin 1-beta (β)
IL-6	Interleukin 6
ISAPP	International Scientific Association for Probiotics and Prebiotics
L. reuteri	*Limosilactobacillus reuteri*
LBP	Live biotherapeutic product
LGG	Lacticaseibacillus rhamnosus GG
LNT	Lacto-N-tetraose
LTA	Lipoteichoic acid
MLP	Moonlighting protein
MRS	Mann-Rogosa-Sharpe
MV	Membrane vesicles
NMR	Nuclear magnetic resonance
NTA	Nanoparticle tracking analysis
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
SCFA	Short chain fatty acids
SEM	Scanning electron microscopy
SIM	Simulated intestinal medium
TNF-α /	Tumor necrosis factor alpha
Treg	Regulatory T-cells
TRPV1	Transient receptor potential vanilloid 1
US	United States

Graphical illustration of the work



The main aim of this thesis work was to improve the understanding of the mechanisms by which probiotic strains interact with the host. Several strains were produced and evaluated in different ways, where the production approach was alternated to increase bioactivity. In brief, Paper **I** focused on biochemical and bioactive characterization of the isolated extracellular membrane vesicles (MV), which were evaluated in three models for host interactions (immunomodulation, pain antagonistic effect, and protection of epithelial integrity). In Paper **II**, multiple strains of *Bifidobacterium longum* were assessed for their ability to stimulate *Limosilactobacillus reuteri* during cultivation, and then further characterized in terms of basic characteristics and carbon source utilization. MV isolated from cultures where *B. longum* had stimulated *L. reuteri* were evaluated in two models for host interactions (immunomodulation and pain antagonistic effects). In Paper **III**, *L. reuteri* was grown and formulated in sucrose prior to drying, allowing for production of bioactive compounds through a process called lyoconversion. The effects of this process were evaluated in terms of growth and stability of the bacterial cells as well as the bioactive compounds produced. The lyoconverted sample and isolated exopolysaccharides produced during lyoconversion were evaluated in a model for immune interactions.

1. Background

1.1 The intestine and its inhabitants

In recent decades, public interest in health has increased and research into human diseases has expanded (Mendlovic et al. 2022). The factors that prevent disease and promote health are not fully understood, and there are numerous factors that affect the outcome of microbe-host interactions. There is mounting evidence that intestinal health has strong impacts on the systemic health of individuals. A staggering 10^{13}-10^{14} bacteria are suggested to comprise the human microbiota, most of which are found in the gastrointestinal tract (Abbott 2016). The species diversity is vast, with hundreds or even thousands of species estimated to comprise the human microbiota (Quigley 2013). These bacterial species together constitute the microbiota and colonization begins at birth. In fact, the gut microbiota is regarded as one of the key elements in the regulation of health. Imbalances in the gut microbiota have been linked to multiple diseases, including obesity, diabetes, inflammatory diseases such as intestinal bowel disease, several types of cancer, and gastrointestinal function disorders. The microbiota produces many well-described metabolites important for host health, such as short-chain fatty acids, but also large numbers of other potentially important effector structures, which can be either secreted or bound to the bacterial membrane. Increased understanding of the role and potential causality of the microbiota and its effector structures in various diseases and disorders can aid in the development of therapeutic strategies based on probiotics (Bull and Plummer 2014; de Vos *et al.* 2022). While colonization of the microbiota begins at birth, the fetus is already exposed to bacterial DNA and antigens during development *in utero* (Turunen et al. 2023). The collection of microorganism genomes found in humans is referred to as the human microbiome and represents another entry point in understanding how bacteria impact human health. As humans and their microbial inhabitants in the gastrointestinal tract have co-evolved, there are many intricate ecological interactions. Studying what happens to gut ecology during health and disease can increase understanding of the bacterial impact on human health. The microbiome field has grown rapidly in recent decades and is helping to understand factors that contribute to health and disease,

while also providing the potential for therapeutic and prophylactic options such as probiotics originating in gut ecology (Cho and Blaser 2012). The impact and suggested health benefits of probiotics have been discussed and summarized by others (Hill et al. 2014; Klaenhammer et al. 2012; Nagpal et al. 2012; Sanders et al. 2019). The global probiotics market was valued at US$ 57.8 billion in 2022, has estimated compound annual growth rate (CAGR) of 8.1%, and is projected to reach an astounding US$ 85.4 billion by 2027 (MarketsandMarkets 2023).

In early life, the microbiota is less diverse and it continues to diversify during infant development, after which it stabilizes during childhood (Bäckhed et al. 2015). The early colonization of the infant originates with bacteria from the mother's vagina, feces, skin, and breast milk (Mueller et al. 2015). In fact, microbiota composition and size change over the lifetime of an individual, *e.g.*, some bacteria are more likely to be present in high abundance in infancy, while others are more numerous in adolescence and adult life (Davis et al. 2020; Agans et al. 2011; Sender et al. 2016). However, an important time in human development is the first 1000 days of life, from conception until the child reaches two years of age. During this time, large parts of the microbiota, cognitive and physiological development, and the immune system are shaped, in processes where bacteria play vital roles (Romano-Keeler and J. Sun 2022). Some bacteria, such as *Bifidobacterium* and *Lactobacillus*, have been shown to have a positive impact on infant development.

Bifidobacterium are early colonizers and play a pivotal role in neonatal development, with absence or reduced levels of bifidobacteria being correlated with later metabolic, immunological, and neurodevelopmental diseases (Saturio et al. 2021). There is also a correlation between bacterial species, phase in life, and diet. For example, *Bifidobacterium longum* subsp. *infantis* is generally more metabolically prepared to metabolize oligosaccharides originating in mothers' milk, while other bacteria, such as *B. longum* subsp. *longum*, more often carry genes involved in plant polysaccharide metabolism, reflecting their age-associated ecological niches (Turroni et al. 2009; Turroni et al. 2012; Kujawska et al. 2020). Lactobacilli are also early colonizers of the infant gut that show up shortly after birth and are continuously supplied to the infant intestine through the breast milk (Servin 2004; X. Zhang et al. 2020). Their presence on the mucosal surfaces of the intestine allows for interactions with host epithelial cells, but also with

the underlying immune cells. Lactobacilli and bifidobacteria have profound effects on health and are important cornerstones of the microbiota by participating in construction of ecological niches, by *e.g.*, oxygen consumption, production of acids, substrate degradation, and direct interactions with the host through *e.g.*, receptor interactions (Walter 2008; Daisley et al. 2021).

1.2 Aims and brief description of Papers I-III

Disturbances in microbial composition in the human gut are evident in the continuous rise of many non-communicable diseases rooted in inflammation, such as obesity, allergy, asthma, and inflammatory bowel disease. Transition or adherence to unhealthy lifestyles is fueling disease and death rates, ultimately leading to lower quality of life and increased healthcare costs. Probiotics are living microorganisms that could act as a potential remedy to disease and a means to promote health. The formal definition of probiotics is "live organisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Several probiotic strains have been well studied and have proven clinical effects. Examples of well-documented strains are *Lacticaseibacillus rhamnosus* GG (LGG), *Bifidobacterium animalis* subsp. *lactis* BB-12, and *Limosilactobacillus reuteri* DSM 17938, among others (Ciorba 2012). Strain LGG has been shown to elicit several health-promoting effects, including prevention and treatment of gastro-intestinal infections and diarrhea (Segers and Lebeer 2014). *Bifidobacterium animalis* strain BB-12 has been shown to increase bowel function and protect against diarrhea, as well as reducing side-effects of antibiotic treatment (Jungersen et al. 2014). *Limosilactobacillus reuteri* DSM 17938, which is one of the strains examined in this thesis, has been proven to be effective in *e.g.*, ameliorating infantile colic (Sung et al. 2017; Szajewska et al. 2014) and treating diarrhea and associated symptoms (X. Sun et al. 2023; Mu et al. 2018).

1.2.1 Selection of suitable probiotic strains

The complexity of the gut microbiota is vast and there is a need to identify mechanisms of action in order to better understand how probiotics can be selected and produced to more efficiently combat diseases and disorders. Selection of a probiotic strain is an important step in the development of

novel probiotics. Finding a strain with an increased ability or desirable trait, or applying selective breeding techniques aimed at promoting these, can be one way of achieving a strain with better bioactivity. Another way of increasing bioactivity (and yield) is by optimizing the production strategy. Examples of improved bioactivity could be increased expression of an enzyme or other structures which interact with the host. One such entity that has been studied intensively in recent decades are extracellular membrane vesicles (MV), which are smaller membrane-encased effector structures that are released into the surroundings and have been shown to have multiple effects and interactions with the host. Assessing strains with different properties in models for host interactions can improve understanding of how clinical effects of probiotics are mediated and of bacterial properties that play a major role in delivering the desired effect.

Therefore, the overall aims of the work in this thesis were to identify how probiotic bacteria interact with each other and with the human host; to assess whether bioactivity can be optimized and improved by selection of strains; and to evaluate stimulatory effects on other strains in the strain selection procedure; and to examine how production of probiotics can be manipulated in order to increase the biological functionality of the bacteria, and thus benefit the host. Specifically, the research aimed at providing insights into *L. reuteri*-derived extracellular membrane vesicle mechanisms and host-interactions (Papers **I** and **II**), investigating *B. longum* BG-L47 and its role as a potential stimulant of *L. reuteri* and associated MV (Paper **II**), and assessing whether industrially applicable production changes can affect the bioactivity of probiotics (Papers **I-III**).

1.2.2 Paper I

The work in Paper **I** involved basic characterization of MV deriving from *Limosilactobacillus reuteri* strains DSM 17938 and BG-R46. We examined their effects in cell models for host interactions aimed at reflecting possible mechanisms for infantile colic, including effects on immune cells, intestinal epithelial cells, and nociceptive neurons. Paper **I** also examined proteomic differences between bacterial cell surfaces and membrane vesicle surfaces. The effects of MV from *L. reuteri* strains DSM 17938 and BG-R46 were compared against those of the well-documented probiotic strain *Lacticaseibacillus rhamnosus* GG.

1.2.3 Paper II

Paper **II** examined the interactions between *L. reuteri* DSM 17938 and *Bifidobacterium longum* BG-L47, and stimulation of growth and membrane vesicle bioactivity by *B. longum*. Paper **II** also assessed characteristics of *B. longum*, including relevant properties important for gastrointestinal passage such as bile- and acid- tolerance and mucus binding. Bioactivity was evaluated with regard to immunomodulatory properties and antagonism of pain signals in nociceptive neurons.

1.2.4 Paper III

Paper **III** studied production of probiotics and assessed whether holding time, *i.e*., the time between two steps in the production process, can affect the final product through production of exopolysaccharides by *L. reuteri* DSM 17938. The products were characterized by chemical methods such as nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GCMS) as well as in cell models of host interactions.

The different bacterial strains used in Papers **I-III** are listed in Table 1.

Table 1. *Strains investigated in Papers I-III in this thesis.*

Genus	Species	Strain	Paper
Limosilactobacillus	*reuteri*	DSM 17938	I, II, III
Limosilactobacillus	*reuteri*	BG-R46	I
Lacticaseibacillus	*rhamnosus*	GG	I
Bifidobacterium	*longum*	BG-L47	II
Bifidobacterium	*longum*	BG-L48	II
Bifidobacterium	*longum*	BB-536	II

2. Probiotics

Consumption of fermented foods, and thus live bacteria, as part of a health-promoting lifestyle is an old idea. In fact, the Roman historian Plinius claimed in 76 B.C that dysentery could be alleviated by drinking fermented milk (Bottazzi 1983). Over a century ago, Henri Tissier of the Pasteur Institute discovered that the microbiota of breastfed infants was to a great extent comprised of bifidobacteria, while the microbiota of formula-fed infants suffering from diarrhea was not (Tissier 1900). Seven years later, when Eile Metchnikoff observed that Bulgarian farmers consuming yoghurt had long and healthy lives (Metchnikoff 1908), the field of probiotics was truly born. Metchnikoff suggested that the longevity and health was due to beneficial bacteria in the yoghurt and other fermented foods. The bacterial species in the yoghurt were mainly *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Metchnikoff 1908). Since then, numerous species, including *Limosilactobacillus reuteri* and *Bifidobacterium longum*, have been shown to exhibit beneficial effects on the host. The first probiotic product was launched in 1935, by Yakult, and contained *Lacticaseibacillus paracasei* strain Shirota. As mentioned earlier, probiotics today are defined as live organisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014). This description covers live bacteria but not postbiotics, which are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al. 2021), placing emphasis on inanimation of the product. The extracellular MV studied in Papers **I** and **II** fits into the postbiotics definition.

For a bacterial strain to be defined as a probiotic, it must meet four major criteria established by the International Scientific Association of Probiotics

and Prebiotics (ISAPP). First, the strain must be adequately characterized, with correct identity and nomenclature. Second, it must be proven safe to consume, either by historical evidence and experimentation, or by Phase 1 studies addressing safety. A regulatory concern in conducting safety studies on probiotics is that most are commonly classified as food supplements and therefore do not have to comply with the stringent rules governing safety studies within the pharmacological industry. However, there are emerging products (a few of which have been approved by the U.S Food and Drug Administration, *e.g.*, Rebyota and VOWST) with live bacteria which fall within the pharmacological group called live biotherapeutic products (LBP). Safety studies addressing LBPs are obliged to follow the rules and legislations of Phase 1-4 studies. However, in the case of probiotics, two regulatory jurisdictions apply to probiotic species and strains within the European Union (EU) and the United States (US), namely Qualified Presumption of Safety and Generally Recognized as Safe, respectively. Third, the probiotic must be supported by at least one human clinical study in which the emphasis is on the health effect achieved by consuming the product. Importantly, the study should be scientifically rigorous, well-designed, and report potential risk of bias. Fourth, the probiotic product must contain sufficient amounts of live bacteria to elicit a health benefit throughout its shelf-life (Binda et al. 2020).

Other criteria have been established with the purpose of aiding in selection of probiotic strains. These criteria begin at isolation and end at clinically proven effect. In brief, they require that the strain is of human origin, lacks pathogenic behavior, is tolerant to processing and stress related to passage through the gastrointestinal tract, can adhere in the intestine, produces antimicrobial compounds, influence and modulates immune responses, and influences metabolic processes. Many of these criteria were evaluated for strain BG-L47 in Paper **II**. A thorough description of all these selection criteria is available elsewhere (Dunne et al. 2001).

While these criteria can help in selection and comparison of probiotic strains, it is important to emphasize that the effects of strains and components observed in preclinical host interaction models may not translate into actual effects in humans. This is a major challenge in describing mechanisms of action, and failure in translation and generalization of preclinical data from various models to effects in humans is a frequent cause of discontinuation in the biomedical research area (Brubaker and Lauffenburger 2020). However,

the use of preclinical models is essential in the field, as they allow for broader screening and better targeted approaches, along with the obvious ethical upsides of conducting experiments *in vitro*. Many types of preclinical models are employed in the research field, including cell lines, primary cells, organoids, organ *ex vivo* models, and finally *in vivo* models, all of which have their respective pros and cons. In Papers **I-III** in this thesis, most of the acquired data on the effects of strains, supernatants, MV, and exopolysaccharides (EPS) were obtained using *in vitro* models. While such models are unable to depict the full picture, they can provide guidance in narrowing down the mode of interaction with the host. When attempting to mimic an *in vivo* situation, the consensus is that primary cells are preferred over immortalized cell lines, due to several advantages such as higher biological relevance and low genetic drift (Aldrich 2023). A model for interaction with the pain receptor transient receptor potential vanilloid 1 (TRPV1) was evaluated as an example in Papers **I** and **II**, since pain is believed to part of the etiology of infantile colic and TRPV1 is one of the main receptors involved in pain perception in the intestine (Burgos et al. 2015). Rat primary dorsal root ganglion cells were used in the model, but a TRPV1-over-expressing human cell line was also evaluated, to complement the main findings and confirm that the antagonistic effects were true in a model of human origin (unpublished data). The model used in the TRPV1 assay is depicted in Figure 1.

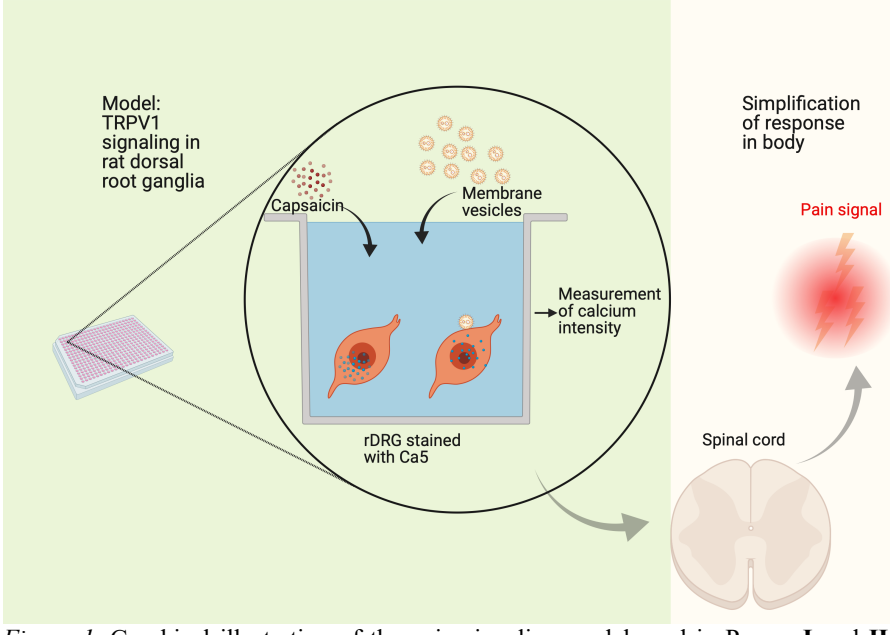


Figure 1. Graphical illustration of the pain signaling model used in Papers **I** and **II**. Primary rat dorsal root ganglion cells were stained with a calcium indicator (Ca5) and incubated with membrane vesicles. Upon stimulation with the TRPV1 agonist capsaicin, release of calcium was measured. Calcium release triggers a cascade of signals that are eventually interpreted as gut pain. The membrane vesicles were shown to antagonize TRPV1, reducing the transmission of pain signals.

2.1 *Bifidobacterium*

Bifidobacteria belong to the phylum *Actinobacteria* and are anaerobic, irregular rod-shaped bacteria with rudimentary branching contributing to their bifid morphology. They are among the first bacteria to colonize the human gastrointestinal tract. Henry Tissier discovered *Bifidobacterium* back in 1899, and later evaluated whether it could act as a remedy for infantile diarrhea (Tissier 1906), showing that the belief of an association between bifidobacteria and health is not new. Indeed, bifidobacteria have frequently been associated with health, probably owing to their over-representation in breastfed infant feces and reported correlations between lower amounts of bifidobacteria and a variety of common disease conditions such as obesity, diabetes, and allergies (J.-H. Lee et al. 2008; Arboleya et al. 2016). The fecal

microbiota of formula-fed infants is also dominated by bifidobacteria, but the relative abundance is approximately 20% higher in the fecal microbiota of breastfed infants (Tannock et al. 2016). Babies delivered by caesarean section have been reported to have delayed colonization by *Bifidobacterium* (Grönlund et al. 1999).

Since their discovery by Tissier, *Bifidobacterium* species have been discovered in several ecological niches, including the oral cavity and gastrointestinal tract of vertebrates (Ventura et al. 2007). The most common *Bifidobacterium* species in the human gastrointestinal tract is *B. longum*, although other species such as *B. breve* and *B. bifidum* are also highly prevalent (Lugli et al. 2023). Interestingly, it has been demonstrated that only three bacterial species, including *B. longum* and *B. breve*, act as markers of stable communities and stabilizing members of the gut microbiota of adults (Olsson et al. 2022). In line with this, Arboleya and co-workers concluded that *B. longum* is one of the dominant species of bifidobacteria in infants, but also in adults (Arboleya et al. 2016), indicating that it may play a vital role in the microbiota.

2.1.1 Ecology

The ecological role of *Bifidobacterium* in the human microbiota is broad. These species contribute substantially to the microenvironment by degrading dietary sugars and fiber into shorter carbohydrates and short-chain fatty acids (SCFA), which can be utilized by other species in the microbiota. Thus, bifidobacteria (along with other species) perform an important first step in sharing bioenergetic resources (Daisley et al. 2021). By doing so, bifidobacteria can benefit other bacterial species with diverse functions. Importantly, during degradation of fiber and sugars, bifidobacteria produce acetate, which is one of the most important SCFA in the intestine as it serves as a substrate for bacteria that produce other vital compounds, such as vitamins and butyrate.

In the infant gut, the sole nutrient source ingested is breast milk, which contains fiber in the form of human milk oligosaccharides (HMO), the third most abundant component in breast milk. These complex carbohydrates are indigestible by humans, but several bacterial species, including *B. longum* subsp. *infantis*, have the metabolic capacity to digest HMOs, which is likely of major importance for their colonization of the infant gut. The reasoning about bifidobacteria as important inhabitants of the healthy human

gastrointestinal tract is fueled further by the fact that the sole source of nutrients in the infant diet contains approximately 5-15% HMO, aimed at benefiting bacterial primary degraders (Walter et al. 2011).

Bifidobacteria are able to metabolize complex fiber through expression of glycoside hydrolases (GH), which are enzymes involved in fiber degradation. A plethora of different glycoside hydrolases are involved in degradation of fibers of different origin, including HMO, host-derived glycans (*e.g.*, mucins), dietary glycans (plant origin), and microbially derived glycans. *Bifidobacterium longum* subsp. *longum*, which was the main focus of the research in Paper **II**, is less well equipped for HMO utilization, but instead has enzymes for plant fiber degradation, which is associated with transitional and later parts of life. In Paper **II**, *B*. *longum* BG-L47 was found to be capable of growing on lacto-N-tetraose (LNT), which is one of the most abundant HMO in human breast milk. While there are previous reports of *B. longum* subsp. *longum* fermenting LNT, this was not a feature of the other two strains evaluated in Paper **II**. The genome of *B. longum* strain BB536, which possesses glycoside hydrolases associated with LNT utilization, was also investigated, but it was found to be unable to grow on LNT as a carbon source. Utilization of LNT may help strain BG-L47 to colonize the infant gastrointestinal tract earlier and more effectively and is potentially also important for continuing colonization as the infant grows older and starts to ingest plant fiber. This does not necessarily mean that BB536 is less well adapted to colonize the infant intestine, but it may be an indication that strains of *B. longum* need to collaborate with other bacteria in order to colonize. In fact, some of the enzymes from bifidobacteria involved in fiber degradation have been shown to be localized extracellularly and products from fiber degradation can be used by other strains (Drey et al. 2022). In Paper **II,** the location of different glycoside hydrolases enzymes in BG-L47 was predicted, with 16 of them predicted to be localized on the surface or secreted (often sortase-dependent, cell wall-anchored proteins with an LPxTG domain). This domain is often found in proteins of importance for host and surrounding interactions (Ossowski *et al.* 2011; Ossowski 2017; Call and Klaenhammer 2013), potentially facilitating an important ecological role in niche construction. The discrepancy between BG-L47 and BB536 in terms of LNT utilization could constitute an example of the Black Queen hypothesis in microbial ecology, which refers to an evolutionary strategy that essentially streamlines the genome and favors

growth over costly genetic material and results in genes being lost (Finn et al. 2022; Daisley et al. 2021). The theory yields a mutual, cooperative, or parasitic behavior among species or strains, where the extracellular bioenergetic resources are shared among species. These shared extracellular bioenergetic resources have been described as the human gut "pantryome" (Daisley et al. 2021). The theory ultimately means that some species or strains can rely on others to carry the genetic burden that comes with metabolizing a substrate, and then supply the rest with energy sources. In the context of this thesis, given that extracellular degradation of LNT is enabled by other species and that LNT and other HMO-derived components are released into the surroundings, *B. longum* BB536 could benefit from being ecologically less burdened. The glycoside hydrolases involved in LNT degradation have been shown to localize extracellularly (Wada et al. 2008), but this needs to be confirmed in future research. Using a simplified simulated intestinal medium with nutrient scarcity and glucose as the carbon source, Paper **II** investigated the effects of stimulation of *B. longum* strains on growth and activity of *L. reuteri* DSM 17938. It would also be interesting to develop a medium in which potential synergistic interactions between different *Bifidobacterium* species and strains could be studied under the influence of different carbon sources associated with infancy or adulthood. That said, 13% of the genes in the *Bifidobacterium* genome are involved in carbohydrate metabolism (Wei et al. 2023), and the inability of BB536 to metabolize infant nutrient-associated fiber could just be an example of strain differentiation. Diversity of glycoside hydrolase genes in different subspecies of *B. longum* has been described in a recent study (Vatanen et al. 2022), where the authors discuss transitional *B. longum*. Based on the facts that BG-L47 can metabolize LNT and has a broad repertoire of plant fiber-degrading enzymes with verified phenotype, one could argue that it has transitional properties. Another interesting question is the impact of different fiber types on *B. longum* physiology and gene expression. Speculatively, the carbon source constitutes an important age-/diet-related signal with an impact on protein expression, stress tolerance, and biological activity.

As mentioned, *B. longum* is also abundant in the adult gut (Arboleya et al. 2016). Paper **II** demonstrated that BG-L47 can grow well on plant-derived fiber compounds such as arabinoxylan, which is commonly found in cereal and consists of polymers made up of arabinose and xylose. This possibly

indicates versatility among some strains of *B. longum* subsp. *longum* and an ability to colonize the gastrointestinal tract of humans throughout life.

2.2 *Limosilactobacillus reuteri*

Limosilactobacillus reuteri (formerly *Lactobacillus reuteri*) belongs to the phylum *Firmicutes* and is a rod-shaped facultative anaerobic, lactic acid bacterium which occurs naturally in the gastrointestinal tract and other mucosal surfaces of warm-blooded vertebrates. It belongs to the genus *Limosilactobacillus*, which was described after reclassification of the previous *Lactobacillus* genus and consists of approximately 30 characterized species (Zheng et al. 2020; https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) The new taxonomic nomenclature includes the prefix 'limosi-', which is derived from the Latin word *limosus*, meaning slimy, based on the characteristic that many species of this genus can produce exopolysaccharides from sugars, a topic investigated in Paper **III**. Unlike bifidobacteria (see section 2.1), lactobacilli generally do not have the ability to degrade polysaccharides or fiber. The main species studied in this thesis was *Limosilactobacillus reuteri*, which was first described as *Lactobacillus reuteri* in 1980 (Kandler et al. 1980) but was in fact discovered in the 1960s by Gerhard Reuter, from whom the name derives. Before Kandler announced the new species classification as *Lactobacillus* reuteri, Reuter called the species "*Lactobacillus fermentum* Biotype II".

The nutrient requirements of *L. reuteri* are considerably stringent and the species requires readily fermentable sugars, amino acids, vitamins, and accessible nucleotides. Sugars and some oligosaccharides constitute the major carbohydrate sources for lactobacilli, *L. reuteri* included, and both are in high abundance in the upper gastrointestinal tract (Gänzle and Follador 2012). However, the type of oligosaccharide utilized strongly affects the growth potential of *L. reuteri*. Since it is a heterofermentative species, *L. reuteri* utilizes the phosphoketolase pathway, converting glucose into lactate, ethanol, and carbon dioxide during fermentation. However, when the phosphoketolase pathway is employed the amount of energy retrieved is rather low, due to poor redox potential (Arsköld et al. 2008). This growth limitation can be ameliorated by supplying *L. reuteri* with external electron acceptors, as they allow alternative re-oxidation of NAD(P)H. Known

electron acceptors for *L. reuteri* DSM 17938 are fructose, 1,2-propanediol, pyruvate, citrate, malate, fumarate, glycerol, and oxygen (Arsköld et al. 2008; Cheng et al. 2020).

2.2.1 Ecology

The strong interest in lactobacilli does not originate from high abundance of lactobacilli in the intestinal tract. In fact, lactobacilli are present at low relative abundance (around 0.01%) (Walter 2008), which corresponds to approximately 10^6 colony-forming units (CFU) per gram feces, although the relative abundance is likely higher in the nutrient-rich small intestine. Rather, the interest arose from the broad use of lactic acid bacteria in production of fermented food, tracing back to Metchnikoff's early conviction that humans should consume foods containing bacteria for a healthy life, and are adapted to do so (Maleki Vareki et al. 2018). Recent findings by large study groups confirm the notion that there are health improvements associated with consumption of foods containing high microbial concentrations (Hill et al. 2023). Lactic acid bacteria and lactobacilli are found in many fermented foods, and also on mucosal surfaces in the human body (Walter 2008). This makes them interesting bacteria that undoubtedly interact and influence human cells, with implications for health (Marco et al. 2021). However, in the context of gastrointestinal ecology lactobacilli species do not appear to be correlated with ecological stability, but rather with a fluctuation of the microbiota. Not all lactobacilli species are true inhabitants of the mammalian intestine; Jens Walter neatly depicted that specific species of lactobacilli are autochthonous to the mammalian intestinal tract, while others are not (Walter 2008). This may begin to explain why specific strains of lactobacilli are successful probiotics that colonize transiently, accurately interact with specific targets rather than affecting the whole microbiota, and then exit. This would also support the notion that low concentrations of a non-colonizing probiotic can exhibit effects on the host in a vast ecosystem containing 10^{13} bacterial cells. Interestingly, one of the species that has been found to be autochthonous in some vertebrates is *Limosilactobacillus reuteri* (Oh et al. 2010; Duar et al. 2017). It would seem as though *L. reuteri* has a long-lasting relationship with warm-blooded vertebrates and different strains have been isolated from a variety of animals, such as pigs, chickens, rodents, and humans. Another interesting finding by Walter and co-workers when

assessing joint evolution of *L. reuteri* strains isolated from pigs, chickens, and rodents was that *L. reuteri* strains derived from vertebrate species are faithful to the host species which they colonize and in which they reside (Oh et al. 2010). However, this is not the case for human-derived *L. reuteri* species (Duar et al. 2017). Through ancestral state reconstructions, Duar *et al.* (2017) found that *L. reuteri* from poultry and humans share ancestral history. *L. reuteri* still colonizes the gut of humans in some Western countries (Sinkiewicz and Ljunggren 2009; Abrahamsson *et al.* 2009).

Effects of industrialization

The finding that *L. reuteri* from poultry and humans share ancestral history raises the question of whether and why the human gastrointestinal tract exhibits greater colonization resistance than that of other vertebrates (Duar *et al*., 2017; (Dalby and Hall 2021). This could be associated with the effects of industrialization and dietary changes that have arguably had a major impact on the human lifestyle. For example, it has been demonstrated that *L. reuteri* colonizes the gastrointestinal tract of Papua New Guinean populations in rural areas, who are less exposed to the lifestyle changes associated with industrialization, but does not colonize that of the US population (Martínez et al. 2015). Studies dating back to the 1960s have routinely detected *L. reuteri* in Western populations (Walter et al. 2011) and the occurrence of the species is still rather high in some countries and populations, ranging from 0% to 50% (Sinkiewicz and Ljunggren 2009). Interestingly, one study found that *L. reuteri* was highly prevalent in Swedish infants regardless of probiotic or placebo treatment (Abrahamsson et al. 2009). Martínez and colleagues identified several modern lifestyle factors that play a major role in development of the microbiota, including diet, formula or breastfeeding, and cesarean section (Martínez et al. 2015). *Limosilactobacillus reuteri* cannot metabolize plant-derived fiber, with the exception of the oligosaccharide raffinose (Z. Zhang et al. 2020). Together with utilization of raffinose and other readily available sugars, the species relies on other inhabitants of the microbiota, such as *Bifidobacterium*, to perform primary degradation of fiber. In the study by Martínez *et al*. (2015), alpha diversity was lower in the US cohort than in the Papua Guinea cohort, which could be a result of the lower fiber consumption in Western societies compared with non-Westernized populations. Likewise, it has been demonstrated that a diet with low concentrations of fermentable

oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) impacts the microbiota and reduces the total bacterial abundance of butyrate-producing *Clostridium* cluster, as well as mucus-associated *Akkermansia muciniphila* and fiber-degrading *Ruminococcus torques* (Halmos et al. 2015). An earlier study showed that *Bifidobacterium* was reduced in response to low FODMAP (Staudacher et al. 2012). Paper **II** demonstrated that *L. reuteri* can benefit from co-culture with a supporting *B. longum* in a simplified simulated intestinal medium. A recent study also showed that *L. reuteri* gains increased fitness and is in fact ecologically benefited in gnotobiotic mice by a cross-feeding interaction with *Bifidobacterium*-derived 1.2-propanediol (Cheng et al. 2020). Interestingly, the authors found that the operon encoding the genes for 1.2-propanediol utilization (*pduCDE*) imposes a burden for *L. reuteri*, but that the operon-carrying strain is ecologically benefited in the presence of a *B. breve*-producing propanediol and a mucin-degrading *B. bifidum*. This indicates that the microbial networks in the gut are essential and that loss of one species as a result of lifestyle changes can impact the microenvironment of the intestine and thereby also other species. Analogously, the reduction in primary fiber degraders such as *Bifidobacterium* and *Ruminococcus*, as well as other bacteria with important functions in the human intestine, as a result of a low-fiber diet could impact the ecological circumstances in which *L. reuteri* colonizes the human intestine. Many of the factors identified as being changed by industrialization (Martínez et al. 2015) have profound effects on the microbiota. Another contributing factor to *L. reuteri* being a colonizer of the gastrointestinal tract is its potent effects as an immune modulator, able to affect macrophages and dendritic cells in a way that potentially promotes immunological tolerance to the species (Walter 2008). Interestingly, the enzyme 5' nucleotidase (5'NT) studied in Papers **I** and **II**, which converts adenosine monophosphate into the potent immune modulator adenosine, has been shown to elicit immune suppressive effects that consequently promote colonization by other bacteria (Soh et al. 2020; Alam et al. 2015)

2.3 Synergism among bacteria

Considering the astounding number of bacterial species in the human gastrointestinal tract, it comes as no surprise that the complexity of the interactions within this community is immense. One could regard the

gastrointestinal tract as an ants' nest, but instead of several different types of ants, with corresponding duties, there are hundreds or thousands of different microbial species. These microbial communities are important for the maintenance of human health. When the structure of the microbiota is perturbed this often results in dysbiosis, which can lead to disease (C. Petersen and Round 2014). Another suggested definition of dysbiosis puts more emphasis on the failure of the bioenergetic resource network and associated disappearance of stabilizing roles within the community, which ultimately affects extracellular resource sharing (Daisley et al. 2021). Causal factors affecting the microbiota and possibly leading to dysbiosis have been thoroughly described by others but are generally associated with industrialization of the modern world. From an evolutionary perspective, industrialization and associated factors such as use of antibiotics, sanitary measures, processed foods, and medical advances like cesarean section are completely new, and their effects on gut microbiota have been neglected (J. L. Sonnenburg and E. D. Sonnenburg 2019). The lifestyle changes associated with industrialization that have altered the microbiota have coincided with the emergence of inflammation-driven diseases. It was only recently recognized that global industrialization has brought about potentially irreversible changes in the microbiota, with possible roles in chronic inflammatory diseases (E. D. Sonnenburg and J. L. Sonnenburg 2019; J. L. Sonnenburg and E. D. Sonnenburg 2019). This tremendous microbial diversity of the human gastrointestinal tract indicates that there are intricate mechanisms between the bacteria that yield a congregation of ecological niches. These niches are probably shaped by physiological and immunological circumstances of the host. They are also shaped by development and maturation of complex food webs, where one bacterial species facilitates the growth of another by producing a product that functions as a substrate for the other (Walter 2008). A simplified way to investigate bacterial interactions and potential food webs between species is to set up a cross-feeding experiment, where one of the bacteria is capable of utilizing the substrate and the other is not. For example, primary degraders such as *Bifidobacteria* can break down fiber into smaller sugar components that subsequently stimulate the growth of non-fiber metabolizing bacteria, some of which produce short-chain fatty acids in response, ultimately benefiting the host (De Vuyst and Leroy 2011; Silva *et al.* 2020).

2.3.1 Bifidobacteria and lactobacilli

While carbon source plays a major role in the growth of bacteria, other critical components, such as electron transfer, also require consideration. Intricate symbiotic mechanisms between bifidobacteria and lactobacilli have been described, where *B. breve* produces 1,2-propanediol which serves as an electron acceptor for *L. reuteri* (briefly reviewed in section 2.2.1). In Paper **II**, the limiting factor was not access to sugars, but rather the need for another stimulating factor, *e.g.*, an electron acceptor. In the paper, the stimulatory potential of *B. longum* during cultivation of *L. reuteri* DSM 17938 was assessed in a simplified simulated intestinal medium (SIM), in which *L. reuteri* is unable to grow without the addition of an external electron acceptor. Interestingly, it was found that presence of *B. longum* in the medium allowed *L. reuteri* to grow. It was known prior to the work in Paper **II** that cells of *B. longum*, but also the cell-free supernatant, stimulate growth, indicating that the component which *B. longum* produces is secreted into the extracellular surroundings. Given that *L. reuteri* grew in the presence of this compound, the structure was postulated to have electron-accepting properties. As such, it fits well into what has been described as the 'pantryome', contributing to sharing of bioenergetic resources other than by cross-feeding (Daisley et al. 2021). It was also known in advance that *B. longum* would not grow in the SIM and that the molecule is probably produced while the cells are not growing, but metabolically active. By fractionating the workflow, three samples were obtained (medium prior to any bacteria, medium in which *B. longum* had incubated for 48 h and said medium in which *L. reuteri* had grown) and were compared thoroughly using NMR. The results revealed one evident peak at δ_H 1.83 ppm which was not present in the medium prior to bifidobacteria addition, was present in the intermediate sample, and disappeared after *L. reuteri* growth. Comparison of the different fractions with all known electron acceptors used by *L. reuteri*, including fructose, pyruvate, citrate, nitrate, malate, fumarate, glycerol, oxygen and 1,2-propanediol (Arsköld et al. 2008; Cheng et al. 2020), revealed that none of these metabolites were a match.

2.3.2 The importance of *Bifidobacterium*

As mentioned, most bifidobacteria are strict anaerobes and prominent colonizers of the human gastrointestinal tract, especially during infancy. While the oxygen concentration diffusing into the lumen of the adult intestine is between 0.1% and 1% (Schwerdtfeger et al. 2019), the infant intestine is more oxygenated than that of adults (Singhal and Shah 2020). This ultimately means that strains of *B. longum* could be ecologically favored by improved oxygen tolerance. Another alternative would be to partner with other bacteria with high oxygen consumption, such as *Streptococcus thermophilus*, which has been postulated as an approach for protection of *Bifidobacterium* species in yoghurt (Lourens-Hattingh and Viljoen 2001). A major drawback to combining *B. longum* with *e.g.*, *S. thermophilus* is the subsequent production of acids and decrease in pH, which would ultimately inhibit the more pH-sensitive bifidobacteria (Lourens-Hattingh and Viljoen 2001; Talwalkar and Kailasapathy 2004). Lactic acid bacteria may also be efficient oxygen consumers, which could support the anaerobic bifidobacteria (Berstad et al. 2016). Interestingly, the pH of the infantile stool has increased over the past century, from pH 4.88 in 1926 to pH 6.5 today (Duar et al. 2020). During the same period, there has been a consistent decrease in *Bifidobacterium* species abundance in the infant intestine (Henrick et al. 2018). Interestingly, a study assessing correlations of bacterial taxa with fecal pH found that only *Bifidobacteriaceae* was associated with a reduction in pH (Henrick et al. 2018). This indicates that production of acidic end-products is an important attribute of bifidobacteria (Henrick et al. 2018) and in the maintenance of intestinal pH (M. A. G. Hernández et al. 2019). In parallel with loss of bifidobacteria and higher fecal pH levels, the prevalence of inflammatory diseases has been increasing globally, and increased oxygen tension in the intestine has been suggested as a potential cause of dysbiosis and subsequent disease (Rigottier-Gois 2013). As explained previously, *L. reuteri* has decreased in abundance in the human gut, but was a more abundant member of the Western microbiota in the 1960s and is still common in the gut of rural populations in Papua New Guinea (Martínez et al. 2015). As also mentioned previously, a potential reason for the observed changes could be that the ecological niches in which bifidobacteria and lactobacilli thrive are severely affected by the industrialized lifestyle (E. D. Sonnenburg and J. L. Sonnenburg 2019; J. L. Sonnenburg and E. D. Sonnenburg 2019). This ultimately results in species loss-related acidic

secondary metabolites, causing pH to rise. Continuous loss of ecologically important bifidobacteria would have downstream effects on other bacterial species, such as butyrate producers (Daisley et al. 2021). Paper **II** evaluated the effects of *B. longum* on the growth and activity of *L. reuteri* DSM 17938 and showed that features other than growth are also affected, including the bioactive properties of the extracellular MV. In an additional clinical safety study performed using *B. longum* BG-L47, there was no increase in the abundance of bifidobacteria in any of the treatment groups (Paper **II**). The participants in the trial were all healthy adults, and in a healthy intestine it is probably desirable to exhibit transient colonization resistance with no or few changes in response to increased intake of *B. longum* BG-L47. However, the study was rather small and no post-study samples were taken, so no conclusions can be drawn on the topic of colonization. Meanwhile, changes in the abundance of *Bifidobacterium* in an efficacy study with *B. longum* BG-L47 addressing *e.g.*, dysbiotic intestines or infant intestines remain to be elucidated.

An alternative to using *e.g.*, *S. thermophilus* to reduce the intestinal oxygen concentration, and thus benefit bifidobacteria, can be deduced from studies addressing the abundance of other intestinal inhabitants in the infant gut. In fact, in a comprehensive study of 32,277 metagenome-assembled genomes comprising over 80 million genes from early life microbiomes, the five most represented bacterial species in the infant gut were *Escherichia coli*, *Enterococcus faecalis*, *Bifidobacterium longum*, *Staphylococcus epidermidis*, and *Bifidobacterium breve* (Zeng et al. 2022). *Escherichia coli* is one of the most common species in the intestine during infancy and later, and this may have ecological implications since *E. coli* utilizes mixed acid fermentation, where oxygen is rapidly consumed as part of aerobic respiration (Förster and Gescher 2014). During mixed acid fermentation, *E. coli* produces end-products such as 2,3-butanediol, which is less acidic than lactate. It is possible that *E. coli* has a symbiotic relationship with bifidobacteria in infants, consuming oxygen, leaving acidification to the bifidobacteria, but also supplying carbon dioxide, which is pivotal for proliferation of bifidobacteria (Clark 1989; Van der Meulen et al. 2006; Kawasaki et al. 2007). During evaluation of *B. longum* in Paper **II**, attempts were made to flush the medium with nitrogen, in order to relieve some oxygen pressure on *B. longum* during growth, but it quickly became apparent that it could not grow in recently flushed medium. Further testing showed

that flushing and resting the medium for a couple of days promoted growth of *B. longum*. This was attributed to carbon dioxide being lost due to the flushing, but diffusing back after storage (Clark 1989; Van der Meulen et al. 2006).

An important metabolite produced by bifidobacteria is acetate. This is the most abundant short-chain fatty acid in the colon and, apart from its many direct functions on the host, it also serves as a substrate for many species in the microbiota (Moffett et al. 2020). Acetate has been shown to affect the microbiota in various ways, including acting as a substrate and thus stimulating the growth of commensal butyrate producers such as *Fecalibacterium, Ruminococcus*, and *Roseburia,* and facilitating cross-feeding and subsequent growth of butyrogenic species on substrates that are otherwise unusable. Furthermore, acetate is inversely associated with bacterially derived toxins interfering with host metabolic functions (Daisley et al. 2021).

2.4 Production of probiotics

To date, the focus in probiotic production has been on achieving high CFU counts and good stability, but there is an ongoing transition to generating products that survive and produce bioactive compounds during transit through the gastrointestinal tract, and thereby confer a beneficial effect on the host. The physiology of bacteria is strongly affected by the method of cultivation, including fermentation medium design, fermentation parameters, and downstream processing. The process ends in lyophilization and long-term preservation, where the aim is to maintain properties of the bacteria. When probiotic bacteria are produced, survival through the gastrointestinal tract can be estimated with different models of exposure to substances encountered in the tract, such as stomach juice and bile acids. Similarly, biological activity can be gauged by measuring enzymatic activity or production of certain compounds. In this thesis, novel strains of *Bifidobacterium longum* were characterized with regard to low pH and bile acid, as well as estimated potential stimulatory probiotic activity of *L. reuteri* DSM 17938 by measuring 5’ nucleotidase (5’NT) activity. However, these are good estimates at best, since the biological circumstances once a probiotic product is ingested are very different, and the actual effect of probiotics only emerges in clinical studies. Studying how different

cultivation approaches affect these attributes can increase the chances of live and vital bacteria reaching interaction sites in the intestine.

2.4.1 Substrate design

Production of probiotics encompasses many steps, posing a risk of changes that could ultimately impact the viability, physiology, and bioactivity of the bacteria. Depending on the bacterial species and strain produced, the production method can differ substantially, which is often attributable to the specific metabolic, physiological, and basic requirements of the target organism. Fermentation substrate typically contains a sugar (which serves as a carbon source), yeast extract (which mainly serves as a nitrogen source), and many other ingredients, including mineral salts and water (Bonnet et al. 2020). The vast majority of the bacteria found in the human gastrointestinal tract are anaerobic, and therefore the fermentation media used for such bacteria, such as *B. longum*, typically contain antioxidants which allow many anaerobes to survive or even grow under less anaerobic conditions. Parts of the production process for probiotics are covered in this thesis, while a detailed description of industrial production of probiotics can be found elsewhere (Fenster et al. 2019).

Fermentation medium affects many aspects of probiotic culture, including growth rate, morphology, physiology, survival to downstream processes, transit through the gastrointestinal tract, and even bioactivity. This was evaluated in Paper **III**, where it was found that growth on glucose or sucrose had an impact on the ability of the bacteria to metabolize and convert sucrose into exopolysaccharides and other bioactive metabolites in the freeze-drying protectant solution. While glucose is the most abundant monosaccharide in the intestine and a preferred carbon source for lactobacilli, it has also been reported to repress metabolic pathways, thus rendering the bacteria less adaptive to alternative carbohydrate sources (Barrangou et al. 2006) and less bioactive (Duboux et al. 2021). This is interesting, as it suggests that the best ecological adaptiveness would be to optimize metabolism towards the most abundant and likely encountered carbon source, glucose, and thus grow the bacteria on glucose. However, one could argue that growth on glucose renders bacteria susceptible due to repression of genes involved in alternative carbohydrate utilization. Mimicking the ecological niche and supplying the medium with components

that will be available *in vivo* might improve the performance of the bacteria. One such approach was demonstrated in a previous study where addition of mucin increased the mucus-binding capacities of *L. reuteri* (Jonsson *et al*., 2001). Given that glucose represses the genes involved in carbohydrate metabolism, it is not unlikely that other ecologically relevant sugars may promote probiotic performance. In preliminary evaluations following Paper **III**, galactooligosaccharide (GOS) containing lactose appeared to improve bile and acid tolerance of *L. reuteri* DSM 17938 (unpublished data).

2.4.2 Fermentation parameters

Once the basic nutritional needs are met by the growth medium, other cultivation parameters need to be addressed. There are many environmental factors that could affect the outcome of fermentation and the physiology of the bacteria, including temperature, agitation, oxygen, carbon dioxide, inoculum size, *etc*. Alteration of these parameters has been shown to affect morphology, stress tolerance, and freeze-drying survival of *L. reuteri* DSM 17938 (A. Hernández et al. 2019; Rao et al. 2023).

Fermentation optimization could supply the bacteria with nutrients in abundance and avoid potential stressors based on metabolic and physiological features of the strain, thus achieving high yield. However, this approach often yields bacteria susceptible to relevant stressors associated with downstream processing, long-term preservation, or passage through the gastrointestinal tract. Therefore, applying a more stringent approach with suboptimal growth conditions has been suggested, potentially hampering growth but achieving tolerant bacteria (Bisson *et al.* 2023; Lacroix and Yildirim 2007).

2.4.3 Downstream processing, freeze-drying and shelf-life

Fermentation is followed by downstream processing of the culture, which often involves concentration of the bacteria and separation of cells from the culture supernatant. This is typically performed by centrifugation or filtration at temperatures around 4 °C, potentially constituting a stressful step in production. Once concentrated and suspended in a lyophilization protectant, the bacteria are often dried in order to achieve long-term preservation and storage stability (Wendel 2021). This is commonly performed by subjecting

the bacteria to freeze-drying, but spray-drying, air-drying, and vacuum-drying are also used. Long-term preservation procedures involve severe stress for the bacteria, which the protectant solution is meant to counteract. Freeze-drying is the most common method, and the stress is mainly thermal, osmotic, oxidative, and mechanical shear by pressure. During downstream processing and freeze-drying, probiotic bacteria will lose a significant amount of the bioactive molecules, such as MV and exopolysaccharides, formed during fermentation (Papers **I-III**), much of which end up in the discarded fraction. An intriguing prospect that would possibly yield a more potent end-product would be to produce a product that encompasses the full fermentation fraction. Another approach was demonstrated in Paper **III**, where *L. reuteri* DSM 17938 was allowed to incubate in the freeze-drying protectant, producing bioactive exopolysaccharides along with other metabolites. This incubation severely affected freeze-drying survival and shelf-life, which would pose a challenge if applying this method in industrial production. However, holding times during production are common (Fenster et al. 2019) and conversion may occur during holding. This indicates that conversion during holding times may need to be monitored and standardized, or that the issue of survival and stability may be important to overcome. More complex freeze-drying protectant formulations have shown promising results and could possibly prevent poor survival (Chen et al. 2023).

2.5 Transit through the gastrointestinal tract

The ultimate goal when producing probiotic bacteria is for the organism to survive passage through the gastrointestinal tract and transiently colonize the intestine, allowing it to interact with the host and thus potentially elicit a positive effect. Modifications and strategies employed during fermentation and downstream processing are means to achieve this goal. Comprehensive reviews on the transit of probiotics through the gastrointestinal tract have been published (Han *et al.* 2021; Mendonça *et al.* 2022) and only parts of the transit journey and associated changes are described and discussed in this thesis.

Transit through the gastrointestinal tract is a daunting task for bacteria (Wendel 2021). Once a probiotic is ingested orally, it will first be exposed to the slightly acidic saliva, which among other components contains salivary

mucins and enzymes such as amylases and lysozymes. The saliva also contains secretory immunoglobulins which prevent pathogenic bacteria from adhering to the oral mucosa and teeth (Marcotte and Lavoie 1998). These are all potential stressors for the probiotic bacteria. However, the exposure time of the bacteria to the saliva is short and studies have demonstrated that the impact of saliva on probiotic survival is very low or even non-existent (Charnchai et al. 2016).

After passing through the oral cavity, the probiotic bacteria reach the stomach, which is a major obstacle in their journey towards the colon and small intestine. The gastric juice is characterized by acidic pH, which poses severe stress due to denaturation of proteins and acidification of the cytoplasm of bacterial cells. This acidic environment varies in intensity, *e.g.*, fasting state pH generally ranges from 1.5 to 2 in humans (Fujimori 2020). The stomach pH fluctuates with food intake and can reach values up to pH 6, but returns to the fasting state level within two hours (Dressman et al. 1990). Gastric emptying time varies greatly depending on what is digested, *e.g.*, the stomach is more rapidly emptied of liquids than of solid foods. Solid foods may take up to three hours before the stomach is considered emptied (Hellmig et al. 2006). In a study measuring gastric residence time of a radio-transmitting pH-sensitive capsule, the median value was found to be 1.1 hours in healthy children (Fallingborg et al. 1990). This variation in exposure time raises questions about the most suitable pH and residence time for a model aiming to mimic the acidic environment that the bacteria encounter in the stomach juice. In Paper **II,** pH 3 and exposure time of 90 minutes were selected, since these have been established to be the most appropriate acid tolerance measurement conditions (Ko et al. 2022) and have been used previously for evaluation of *B. longum* in gastric pH tolerance tests (Toscano et al. 2015). For lactobacilli, which are less sensitive to low pH than bifidobacteria, simulated gastric juice with a lower pH is often used (Wall et al. 2007; Cele et al. 2022). Another aspect of gastric pH survival of probiotics is whether these should be taken with food or between meals (fasting stomach). The gastric stress for the bacteria will be significantly reduced if they are taken with food, as there will be a pH-buffering effect of the food. However, the gastric emptying time increases with ingestion of food, which means that the exposure to a slightly acidic to very acidic environment will be prolonged compared with taking a probiotic on an empty stomach.

The next major obstacle during the journey of a probiotic through the gastrointestinal tract is exposure to bile salts, pancreatin, and lysozyme. Bile salts are synthesized in the liver by cholesterol conversion, creating bile acids. Bile acids are then conjugated with the amino acids glycine or taurine, creating conjugated bile acids which are stored in the gall bladder until triggered for release into the duodenum upon ingestion of food (Ruiz et al. 2013). Bile acids are detrimental to bacteria, as they disrupt the bacterial membrane and can cause DNA damage, which is why bile tolerance is often considered an important trait of probiotic strains. The ability to deconjugate bile salts via bile salt hydrolase (BSH) is sometimes described as desirable, due to the contribution to bile resistance and reduction in serum cholesterol levels (Foley et al. 2021; Hernández-Gómez et al. 2021). However, whether BSH activity and subsequent deconjugation of bile acids contributes to survival of lactobacilli and bifidobacteria in the intestine remains unclear, as the unconjugated bile acids are more hydrophobic and potentially toxic due to their ability to freely pass over the bacterial membrane. As for lysozyme and pancreatin, the stress is rather mild and does not affect viability to a great extent, and mapping of resistance to lysozyme and pancreatin is rarely performed for probiotic strains (Turchi et al. 2013; Charnchai et al. 2016). Paper **II** evaluated bile tolerance for three *B. longum* strains, and the results showed that strains within the same subspecies can differ substantially. Another observation in Paper **II** was that batch-to-batch variations in assays utilizing biological substances such as bile can be large, as also reported by others (Wendel 2021). Careful consideration and documentation are required in terms of substrate origin, manufacturer, type, and batch, as differences in these parameters can yield different results. An overview of the steps from fermentation to administration is presented in Figure 2.

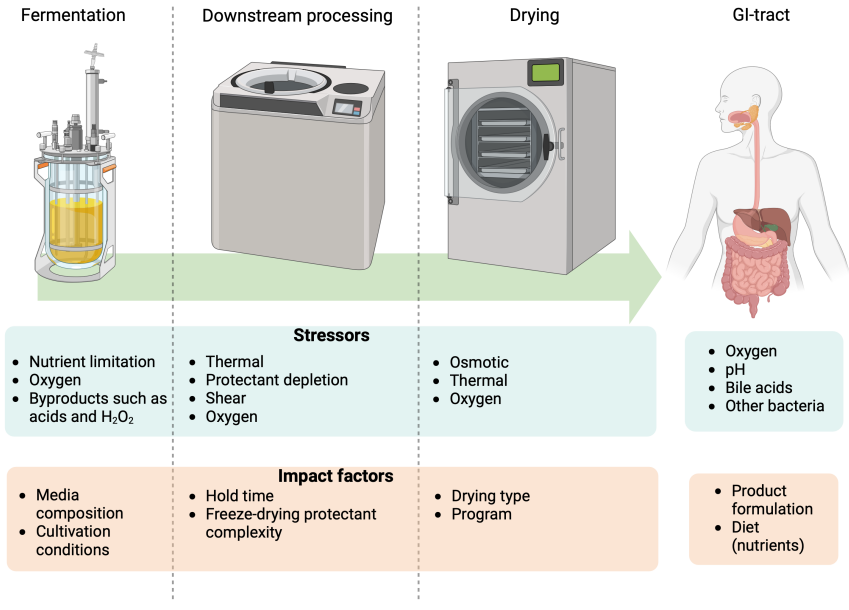


Figure 2. Stressors and impact factors associated with production of probiotic bacteria and subsequent transit through the gastrointestinal tract. By altering any of the parameters listed under 'impact factor, it is possible to obtain more vital, tolerant, and bioactive probiotic bacteria which can be evaluated with regard to any of the stressors. The ultimate aim of producing stress-tolerant bacteria is to improve their survival in the gastrointestinal tract and ultimately allow them to interact with the host.

2.6 Summary

Bifidobacteria and lactobacilli are important inhabitants of the gastrointestinal tract, with well-documented positive effects on the host. *Bifidobacterium longum* and *Limosilactobacillus reuteri* are two species which are autochthonous to the human gastrointestinal tract but appear to be declining in Westernized societies. A complex interplay between bacterial species in the intestine is a prerequisite for successful colonization, and the microbial population is reliant on evolutionary niche construction. Producing probiotics is a complex task, where many stressors must be considered in order to produce robust and potent probiotics with the goal of reaching the intestine alive.

3. Bioactivity

Much of the work in this thesis focused on the bioactivity of probiotics and their derived effector compounds. Lactobacilli and bifidobacteria are known to produce a variety of different compounds and structures that can have a beneficial effect on the host, and thereby promote health. The effects of probiotics have been widely studied, but considerably less is known about specific mechanisms by which probiotic bacteria promote health and prevent disease (Daliri et al. 2021). Bioactive compounds and structures (bioactives) can either be located on the surface of the bacteria or released into the surroundings. As described in section 2.4 of this thesis, probiotic bacteria may produce and secrete large quantities of these bioactives during cultivation, but they may be later lost during downstream processing in the production of probiotics. How the production of these structures in specific strains is affected by cultivation method has been explored by others (Madjirebaye et al. 2022; Alizadeh Behbahani et al. 2020). However, what truly matters is production of bioactives in the intestine, which is more difficult to address. Bioactives in the intestine have been reviewed (Indira et al. 2019) and include short-chain fatty acids, vitamins and enzymes, among others. Production in the intestine is probably one of the major advantages of probiotics compared with other "biotics". Viability is a prerequisite in the production of probiotics, but with the introduction of postbiotics, defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host", the focus could change to production of bacterial products with maximized bioactivity (Salminen et al. 2021). A non-exhaustive list of probiotic-derived effector structures and compounds relevant to this thesis is presented in Table 2.

Table 2. *Non-exhaustive list of probiotic-derived effector structures and compounds relevant for this thesis*

Bioactive compound	Effect	Strain	Reference
Extracellular membrane vesicles	Pain antagonism, immunomodulatory, protection of epithelial integrity, modulation of gut motility	DSM 17938, BG-R46	Paper **I,** Paper **II**, (West et al. 2020)
Exopolysaccharides	Protection of epithelial integrity, reduction in proinflammatory cytokines	DSM 17938	(Kšonžeková et al. 2016), Paper **III**
5'nucleotidase (adenosine)	Reduction in multi-organ inflammation in T_{reg}-deficient mice.	DSM 17938, BG-R46	(Yuying Liu et al. 2023)
Short-chain fatty acids	Reduction in inflammation, immune modulation	Multiple species and strains	(LeBlanc et al. 2017; Yao et al. 2020)
Lipoteichoic acid	Reduction in inflammatory responses	*Lactobacillus plantarum* A3, *Lactobacillus reuteri* DMSZ 8533, *Lactobacillus acidophilus* CI CC 6074	(Lu et al. 2022)

Other bioactive compounds in addition to those listed in Table 2 include vitamins, bacteriocins, amino acids, amino acid derivatives, and specific enzymes. These are not discussed further in this thesis, but have been thoroughly reviewed by others (Indira et al. 2019). Papers **I-III** showed that *L. reuteri* strains can produce potent effector structures, such as extracellular MV and exopolysaccharides, which were evaluated in models for host interactions. Paper **I** also showed that MV constitute a package of known and tentative bioactives, and that their small size (compared with the bacteria) may increase their motility and bioavailability. Interestingly, MV can translocate and carry enzymes which could be active at distant sites in the body, thus constituting not only a ligand but also a factory of bioactives, such as 5' nucleotidase (generating adenosine) and glucan sucrase (generating reuteran).

3.1 Extracellular membrane vesicles

Secretion of cytoplasm and cell contents into the extracellular surroundings is fundamental for interactions with other organisms and the environment (Sartorio et al. 2021). Work leading to the discovery of microparticles, and membrane vesicles (MV), secreted by cells dates back to the 17th Century and studies of blood coagulation. The story of how findings of fiber filaments in 1660s led to the discovery of "platelet dust" (later re-named microparticles) in the 1940s has been reported by others (Hargett and Bauer 2013). Today, extracellular MV are well-known and described as lipid bilayer nanoparticles which are secreted into the extracellular space by all living cells (Krzyżek et al. 2023). In the field of bacterial MV, there was an initial discovery in 1965 where an auxotrophic *E. coli* was shown to secrete free lipopolysaccharides (LPS) under certain conditions (Bishop and Work 1965). It was later demonstrated that these supposedly free lipopolysaccharides were anchored to membrane structures, which were shown to be bacterial outer MV. In Gram-positive bacteria, MV (MV) were only discovered 30 years later, due to the belief that the cell wall was too robust for MV to protrude without disrupting the cell. Altogether, MV are inanimate, unable to replicate, and have been found to mediate various functions critical for cell-cell interactions. Bacterial MV are known to carry a wide repertoire of messaging cargo, including DNA, RNA, proteins, and polysaccharides (Yue Liu et al. 2018).

3.1.1 *Limosilactobacillus reuteri* DSM 17938-derived membrane vesicles

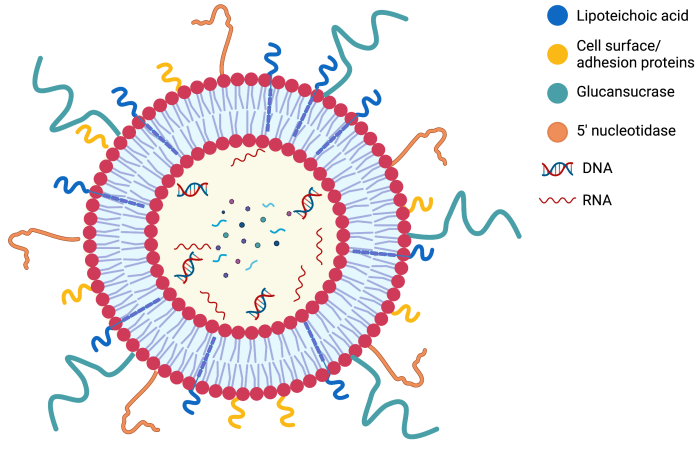


Figure 3. Illustration of *Limosilactobacillus reuteri* DSM 17938- and *L. reuteri* BG-R46-derived membrane vesicles and components found within or on the surface of the secreted particles.

A graphical illustration of MV derived from *L. reuteri* strains DSM 17938 and BG-R46 is presented in Figure 3. The mode of membrane vesicle release follows one of two main pathways, vesicularization of membrane fragments of dead cells or controlled secretion of MV, as distinct from cell death (Toyofuku *et al*., 2017; Brown *et al*., 2015; Orench-Rivera & Kuehn, 2016). Both types of release were observed in Paper **I**, where MV derived from *L. reuteri* DSM 17938 were visualized by scanning electron microscopy (SEM). Oxygen-stressed cells displayed high numbers of vesicles on the surface, while non-stressed DSM 17938 had budding MV of different sizes on the surface. Using proteomics, a group of proteins annotated to be involved in both biogenesis and degradation of the cell wall was identified, indicating that MV are equipped to both open and heal the cell wall. The degradation-associated proteins could potentially also be involved in interactions with other bacterial cells. MV may exhibit competitive and

predatory functions, as indicated by the presence of hydrolases, peptidoglycan hydrolases, and endopeptidases (Díaz-Garrido et al. 2021). There is a need for further research on whether MV can affect pathogen growth and virulence, and thus have an impact on pathogen fitness in the intestine.

It been suggested that MV derived from *E. coli* and *Vibrio cholerae* contain proteins involved in the translation machinery (Sjöström et al. 2015). This indicates a possible route for production of proteins in MV, somewhat blurring the lines between living cells and MV. Interestingly, the proteomics data obtained in Paper **I** revealed more than 50 ribosome-associated proteins among the two *L. reuteri* strains DSM 17938 and BG-R46. Whether endogenous protein production occurs in MV and can be affected by cultivation approaches should be investigated in future research.

Paper **I** demonstrated that MV are potent anti-inflammatory entities derived from *L. reuteri*. Research addressing the ecological role of MV derived from the microbiota and from probiotic bacteria is in its infancy, but there are indications that microbiota-derived MV may play a role in shaping the microenvironment of the intestine by aiding in nutrient acquisition (Díaz-Garrido et al. 2021; Liang et al. 2022). Interestingly, MV derived from a fiber-degrading *Bacteroides thetaiotaomicron* have been shown to carry surface-anchored enzymes involved in fiber degradation (Elhenawy et al. 2014; Valguarnera et al. 2018), which may imply a cross-feeding role of MV. Along with multiple beneficial effects on the host (Paper **I**), MV could be unexplored agents in gut ecology and in shaping of microbiota (Dean et al. 2020). MV from pro- and post-biotics with fiber-degrading capacities may have a role to play in diseases and disorders where high gut fiber content is troublesome, such as irritable bowel syndrome and inflammatory bowel disease. However, much of the work concerning MV derives from *in vitro* culture, and production of MV is altered in response to growth conditions, so *in vivo* production and function of MV require further investigation. A glimpse into the complexity of regulation of membrane vesicle cargo and the impact of bacterial interactions was provided in Paper **II**, where the effects of MV derived from DSM 17938 were boosted by strains of *B. longum*.

On comparing the surface of bacterial cells with the surface of MV from the same strain using surface-shaving proteomics in Paper **I**, it was found that the overlap was only 20-25%, indicating directed protein expression for the MV, which is in alignment with previous findings (McBroom et al.

2006). Thus, while the surface of MV to some extent resembles that of the bacterial cell, they are considerably smaller, potentially making them more mobile. Size is a clear difference between bacteria and MV. The MV derived from Gram-positive bacteria vary widely in size within the range 20-500 nm (Villageliu and Samuelson 2022). In measurements of *L. reuteri* DSM 17938 vesicles using nanoparticle tracking analysis in Papers **I** and **II**, the size rarely exceeded 200 nm. The number of MV produced varies between bacterial strains (Bitto et al. 2021). Moreover, the SEM images presented in Papers **I** and **II** and unpublished images revealed that the number of MV can vary greatly between cells derived from the same culture. There is mounting evidence of heterogeneity among MV, and a species can secrete MV with varying composition (Nagakubo et al. 2019). Quantification of vesicles obtained by ultracentrifugation (derived from the supernatant) using nanoparticle tracking analysis (NTA) was also performed in this thesis and approximately 10^{10} to 10^{11} particles/mL were retrieved, corresponding to approximately 0.1 to 1 vesicle per bacteria. It is worth mentioning that this method measures the MV in Brownian motion, and thus suffers from potential confounding factors in enumeration, such as vesicle aggregation. However, even when adjusted for potential aggregation, the number of vesicles obtained from the analysis can probably be regarded as an underestimate, due to larger vesicles blurring the count of smaller vesicles (Filipe et al. 2010). From rough estimates based on the SEM images, it appeared that each cell had around 20-200 vesicles on the surface, confirming that many vesicles were overlooked in the counting procedure. When isolating MV by ultracentrifugation, the first step was to remove all cells. Many vesicles attached to the cells were probably discarded in the process, which may partly explain the discrepancy. Another reason for the loss of vesicles could be that they attached to surfaces.

In Paper **I**, many effects of *L. reuteri* DSM 17938- and *L. reuteri* BG-R46-derived MV with potential links to the clinical effect were identified (Figure 4). In paper **I,** we also compared these MV with those from *Lacticaseibacillus rhamnosus* GG (LGG) in some of the models for host interactions and revealed strain-dependent effects, where *L. reuteri* DSM 17938- and BG-R46-derived vesicles antagonized TRPV1, whereas LGG-derived vesicles did not. Further, the immunomodulatory effects differed between the strains. Interestingly, the pellet with MV from LGG was translucent and smooth, whereas the pellets with MV from the two *L. reuteri*

strains were white and irregular. NTA showed that the number of vesicles was similar for the strains. Overall, this shows that the physicochemical characteristics and cargo of MV from the two species differ. A recent study also demonstrated that MV from different species and strains are specific (Bitto et al. 2021).

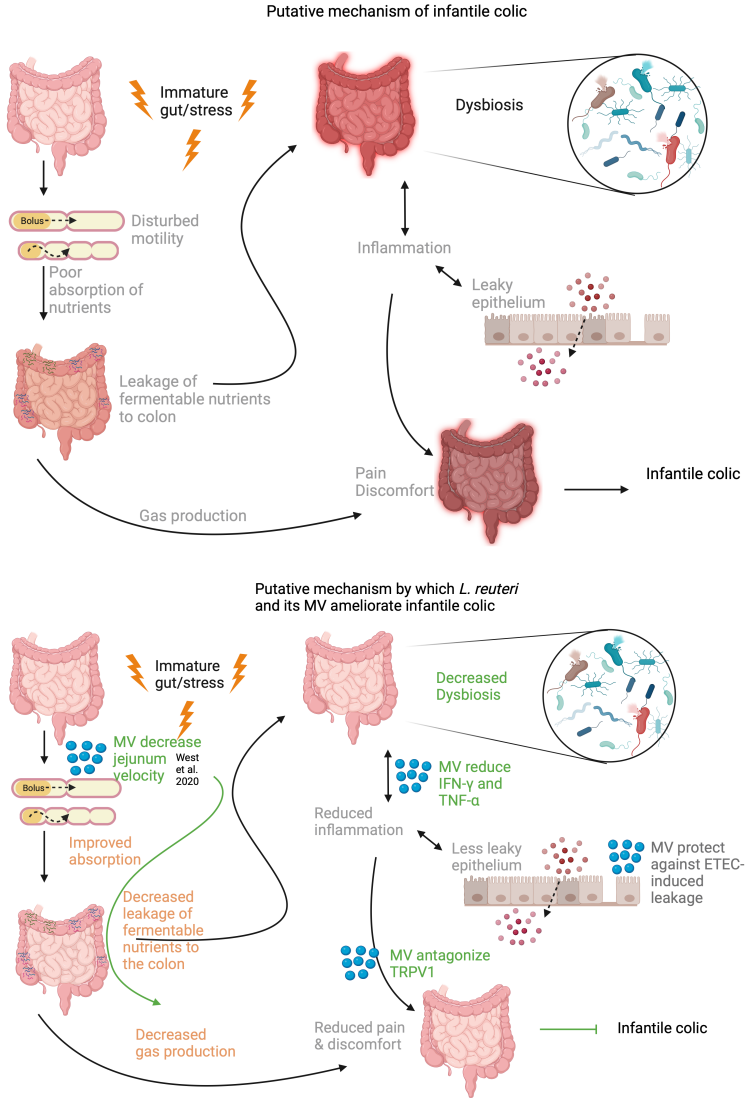


Figure 4. Putative mechanism by which infantile colic occurs (upper) and by which *Limosilactobacillus reuteri* and its membrane vesicles can ameliorate infantile colic (lower).

Most studies to date investigating effects of probiotic-derived effector molecules and bioactive properties of MV have been *in vitro* studies, which lack the physiological complexity of a human gastrointestinal tract. The physiological complexity and presence of functional microbiota are two strong arguments for experiments in animals. However, some of the downsides are that the microbiota of an animal is different from that of humans and that there is a larger variation among individuals, not to mention the ethical considerations that come with animal experiments. It has been suggested that studies addressing the effects of probiotic effectors should aim at mimicking gastrointestinal conditions to the best possible extent (Daliri et al. 2021). To achieve this, in Paper **II** *L. reuteri* and *B. longum* were cultivated in a simplified simulated gastrointestinal medium, from which MV that were evaluated in different models for host interactions, such as immune modulation and pain receptor antagonism, were isolated. The aim was to mimic a situation where the two bacteria interact *in vivo* and increase the likelihood of retrieving MV with similarities to those produced in the gastrointestinal tract.

5' nucleotidase

MV from the *L. reuteri* strains were shown to carry a 5' nucleotidase, or ecto-5'-nucleotidase (5'NT). This enzyme is probably important for the interactions between *L. reuteri* and the host (as discussed in Papers **I** and **II).** It converts adenosine monophosphate (AMP) to the potent signaling molecule adenosine, which has a broad repertoire of functions including immunomodulation, strengthening of barriers, neuromodulation, and pain receptor modulation (Ritchie *et al.* 1997; Ouyang *et al.* 2013; Hong Liu and Xia 2015; Puntambekar *et al.* 2004). Thus 5'NT has similar activity to CD73, an enzyme expressed on multiple cells, among them T_{reg} cells involved in suppression of proinflammatory responses (Alam et al. 2009). *Limosilactobacillus reuteri* strain DSM 17938 has been shown to prolong life and reduce inflammation in mice with T_{reg} deficiency (scurfy mice) (He *et al.* 2017a). A more recent study showed that the effects were attributable to adenosine (Liu et al. 2023). Liu and colleagues showed that *L. reuteri* strains DSM 17938 and BG-R46, which carry the enzyme 5'NT, increased the levels of adenosine as well as the degradation product inosine in parts of the intestinal tract of scurfy mice. Treatment with either of the two strains resulted in elevated levels of T_{reg} cells, but when evaluating a 5'NT-knock-out strain, which doesn't carry the enzyme, the effect diminished (Liu et al.

2023). Moreover, deleting the adenosine A2 receptor in mice has been shown to block the effects of *L. reuteri*, demonstrating that the beneficial effects depend on interaction with that receptor (He *et al.* 2017b). Increasing T_{reg} cells could be fundamental in the alleviation of T_{reg} associated inflammatory diseases. Similarly to *L. reuteri* vesicles, CD 8^+ T-cells have been shown to release $CD73^+$ vesicles that can combat inflammation (Schneider et al. 2021).

Lipoteichoic acid and moonlighting proteins

While parts of the membrane vesicle cargo remain unknown, several components with bioactive properties were identified in Paper **I**, including 5'NT, lipoteichoic acid (LTA) and multiple moonlighting proteins (MLP). Lipoteichoic acid is an important surface structure of Gram-positive bacteria (Selle et al. 2017) and is a microbe-associated molecular pattern that interacts with pattern recognition receptors such as Toll-like receptors (Claes et al. 2012). Lipoteichoic acid differs between species and has been the subject of intense investigation over the years (Jung et al. 2022). It has been shown to elicit bioactive functions, including attachment to host cells (Granato et al. 1999), immunomodulation (Mizuno et al. 2020; Champagne-Jorgensen et al. 2021), alleviation of inflammation (Lu et al. 2022), and anti-biofilm properties (D. Lee et al. 2021). Interestingly, it can also promote inflammation and can even be considered a virulence factor in Gram-positive pathogens (Ginsburg 2002). Further, LTA derived from *L. rhamnosus* GG has been shown to be pro-inflammatory, inducing interleukin-8 in Caco-2 cells (Claes et al. 2012). A study evaluating an LTA mutant of *L. plantarum* in a peripheral blood mononuclear cell (PBMC) model observed a reduction in proinflammatory cytokine secretion in response to the mutant compared with the wild-type strain and also found that the mutant strain was more protective than the wild type in a murine colitis model (Grangette et al. 2005). Altogether, this indicates that improvement of probiotic strains may be achievable through LTA modification. It is worth noting that there are the pleiotropic effects of LTA mutants on cell physiology, for instance altered cell length and flaws in septum formation (Selle et al. 2017; Lebeer et al. 2012). Another suggested role of LTA is acting as an anchor for moonlighting proteins, attaching them to the surface of the bacterial cell and thus allowing them to carry out the moonlighting function, which is often adhesive properties (Kainulainen and Korhonen 2014). Moonlighting proteins are proteins with a cytosolic function which, after secretion, perform another function extracellularly. Many bacterial proteins have been

identified as moonlighting proteins (Jeffery 2018), and an intriguing finding in Paper **I** was that there were many on the surface of the MV. Many of the proteins detected on the surface of the vesicles were annotated to be involved in adhesion (see Supplementary Table S1 in Paper **I**). It has been suggested previously that MV constitute a secretion route for moonlighting proteins (Gil-Bona et al. 2015; Satala et al. 2020) and that LTA may have a supporting function in anchoring to the surface and consequently promoting interactions with the host (Kainulainen and Korhonen 2014).

3.1.2 Isolation of membrane vesicles

In the experiments in this thesis, ultracentrifugation was used for isolation of MV. Ultracentrifugation is often considered the "gold standard" for isolation of vesicles. Density gradient ultracentrifugation yields even purer preparations of ultracentrifugation and is suitable when labeling vesicles for experiments such as microscopy (used in Paper **I** to assess interactions with Caco-2 cells). Ultracentrifugation often results in high yield, but other methods might be more suitable when aiming for high purity (Northrop-Albrecht et al. 2022). These methods include size exclusion chromatography, precipitation, and filtration-based methods such as tangential flow filtration (Northrop-Albrecht et al. 2022; Paterna et al. 2022) and commercial isolation kits. Combinations of methods have also shown promising results (An et al. 2018). However, The suitability of different methods relies on the intended use and research setting (Buschmann et al. 2018). The downside of many commercial kits is the limitation of volume rendering them inapplicable when working with volumes from bacterial cultivations. Tangential flow filtration could be a better solution, as it allows for high volumes continuously flowing through the filter, while also avoiding the shear force of ultracentrifugation and thus achieving less aggregation among the vesicles (Busatto et al. 2018). Others have found that ultrafiltration with subsequent liquid chromatography yields higher numbers of MV with better maintained biophysical properties and biological activity (Mol et al. 2017).

As previously mentioned, during production of probiotics most of the supernatant from cultivation (containing bioactives) is discarded in the downstream processing steps. A potential approach to increase the concentration of bioactive structures in the final formulation was demonstrated in Paper **III**, which showed that the enzyme glucansucrase (present on the surface of both bacteria and MV) was active during

formulation in the lyoprotectant (consisting of sucrose). Without entering the postbiotics field, this type of effector priming could be attempted also with regard to number of MV. *Staphylococcus aureus* has been shown to release smaller amounts of MV in the resting state (Jeong et al. 2022). Factors triggering vesicular release have been studied by many and it has been postulated that release of MV is increased in stressful conditions (Mozaheb and Mingeot-Leclercq 2020). Certain types of stress, including pH variations (A. Hernández et al. 2019; Bodzen et al. 2021) and oxygen level (Rao et al. 2023) have also been shown to increase freeze-drying survival in *L. reuteri* DSM 17938. If some stressors increase vesicle formation and coincide with better freeze-drying, bile or acid survival could have relevance for the probiotic industry, and thus calls for further investigation.

3.2 Exopolysaccharides

Most bacteria grown in the laboratory are planktonic, *i.e.*, bacteria cultivated as single cells or chains in liquid culture. However, planktonic bacteria are rare in nature, *e.g.*, it has been estimated that less than 1% of bacteria in nature occur in planktonic form (Deschênes and Ells 2020). Instead, bacteria often bind to surfaces and aggregate in larger architectural formations, where common function supersedes that of the individual and nutrients are shared, forming multiple niches (Bogino *et al.* 2013; Pereira and Berry 2017). Extracellular polymeric substances form biofilms and one of the main components of these is polysaccharides, which contribute to the architectural form of the biofilm (Bogino et al. 2013). Exopolysaccharides are commonly divided into two groups: *i*) homopolysaccharides, containing one type of monosaccharide, and *ii*) heteropolysaccharides, consisting of two or several types of monosaccharides. Exopolysaccharides from lactic acid bacteria are commonly produced during fermentation of foods, where they improve the rheological properties (Jurášková et al. 2022), and have also been shown to elicit a variety of health-promoting effects (thoroughly reviewed by (Jurášková et al. 2022). Documented effects in response to lactic acid bacteria exopolysaccharides include *e.g.*, immunomodulation (Laiño et al. 2016), cholesterol-removing effects (Gawande et al. 2021), antioxidant capacity, and even reduced polyp formation in a colon cancer rat model (Deepak et al. 2021).

Paper **III** tested the hypothesis that exopolysaccharides can be produced during the holding time between cultivation and freeze drying. An earlier study had already identified and characterized a reuteran (soluble α-glucan) from the mother strain of DSM 17938 (Kralj et al. 2005). On analyzing the genome sequence in Paper **III**, it was discovered that *L. reuteri* DSM 17938 also carries a functional glucansucrase gene, but in addition has a truncated levansucrase gene. The conclusion was that DSM 17398 can produce a glucan, but not a fructan, from sucrose. Furthermore, it has been demonstrated previously that DSM 17938-derived exopolysaccharides protect IPEC-1 cells from enterotoxigenic *Escherichia coli* (ETEC) adhesion and the subsequent inflammatory response (Kšonžeková et al. 2016). Investigation of conversion of sucrose during the holding time (called lyoconversion) in Paper **III** confirmed that an α–(1-4) and α-(1-6) glucan had indeed been formed and in sufficient quantity to elicit a significantly different immunomodulatory response in PBMC compared with an equivalent non-converted sample. Further testing on isolated exopolysaccharides verified the immunomodulatory properties.

3.3 Increasing production of bioactive compounds

As mentioned, probiotic bacteria elicit health benefits by two fundamentally different modes of action, either by modulating the microbiota, and thereby the host, or by transient colonization and interactions with specific targets of host cells. Focusing on the second, the question of how to increase the number of bioactive compounds produced, and thereby improve the probiotic product, was addressed in this thesis.

Linking clinical effects with potential mechanisms of action can provide a better understanding of the attributes that are important for a probiotic strain within the intended use. This knowledge is pivotal and can be a potent tool in the development of new products. During the timeframe of this thesis, several approaches were used in attempts to increase the number of bioactive compounds produced. The approaches included: (*i*) selection, improvement, and combination of strains with increased bioactivity, (*ii*) cultivation protocol optimization, including mild stressors, and (*iii*) allowing and promoting production and secretion of bioactive compounds during probiotic production.

3.3.1 Selection, improvement, and combination of strains with increased bioactivity

In the search for better probiotic products, the first step is to identify a strain with the wanted attributes for the intended use. In Paper **I**, *L. reuteri* BG-R46 was identified as a strain exhibiting greater potency in many aspects that fit the putative mechanism of action for infantile colic (Figure 4). Improved strains can be attained without manipulation of the genome and thus entry into the class of genetically modified organisms (GMO). An extensive review on strain improvement of industrial lactic acid bacteria without using recombinant DNA technology is available elsewhere (Derkx et al. 2014).

As an example, exposing a strain with several desired attributes to a stressful environment can cause naturally occurring mutants with altered characteristics to appear, where the changed characteristics often relate to the stress applied. However, according to the data obtained in this thesis, alterations such as these seldom come singly. Through mechanistic insights into attributes that are desirable for a given purpose, more potent strains can be developed. When the widely used *L. reuteri* strain DSM 17938 was developed, the two undesirable plasmids were removed and the strain also acquired better acid tolerance compared with the mother strain, *L. reuteri* ATCC 55730 (Rosander et al. 2008). Similarly, exposure to bile of *L. reuteri* BG-R46 in Paper **I** resulted in higher 5'NT activity. This enzyme may be involved in the amelioration of infantile colic by multiple interactions between the effector molecule produced (adenosine) and the host. In further experiments, the strain indeed performed better in the epithelial integrity model and in inducing higher levels of cytokine secretions in the model for immune interactions. In the strain selection procedure in Paper **II**, a cryptic plasmid was lost, resulting in *B. longum* BG-L47, a strain with less variable colony morphology.

Another way of improving the bioactivity of a product could be by combining strains in different ways. Growth stimulatory effects of *L. reuteri* provided by *B. breve* have been neatly demonstrated (Cheng et al. 2020). Stimulation of intestinal butyrate-producing *Clostridium butyricum* by lactobacilli has also been demonstrated (SO et al. 2021). These kinds of stimulatory effects could have implications for intestinal ecology, and ultimately human health, through production of beneficial compounds such as butyrate (Canani et al. 2011). To the best of my knowledge, production of bioactive components through co-cultivation of two bacterial species during

fermentation has not been well studied. Paper **II** demonstrated that the activity of MV derived from *L. reuteri* was stimulated by *B. longum* and that the effects were strain specific. The influencing factor provided by *B. longum* remains unknown, but these types of stimulatory interactions merit deeper study in the search for new strains and strain combinations.

3.3.2 Promotion of bioactive compounds by cultivation protocol optimization including mild stressors

When bacteria are exposed to stress, they respond by regulating gene expression, protein activity, and cellular metabolism (Dawan and Ahn 2022). Some of the stress responses can affect production of compounds with bioactive properties. Bacterial membrane vesicle secretion has been shown to increase in response to various stressors, including DNA mutational stress (McBroom and Kuehn 2007), cysteine depletion (van de Waterbeemd et al. 2013), copper oxide nanoparticles, antibiotics, temperature, and hydrogen peroxide (Potter *et al.* 2020; Macdonald and Kuehn 2013). However, one study observed an approximately 100-fold increase in membrane vesicle production in *E. coli* mutants with stress response defects (McBroom and Kuehn 2007). These mutants lacked obvious defects in membrane integrity, demonstrating that the vesicularization was independent of the known stress-response systems that were disrupted.

In Paper **I**, the number of vesicles on the surface of the bacteria was higher for *L. reuteri* DSM 17938 grown under oxygen stress. A recent study investigated whether membrane vesicle production in *L. casei* and *L. plantarum* could be optimized through modifications to the cultivation protocol (Müller et al. 2021). It found that cultivation with agitation and a substrate with pH 6.5 resulted in *L. casei* MV that gave increased IL-10 and reduced tumor necrosis factor-α (TNF-α) expression in macrophages. For *L. plantarum* vesicles, substrate adjusted to pH 5 gave the best anti-inflammatory properties of the vesicles (Müller et al. 2021).

3.3.3 Production of bioactive compounds by lyoconversion

Paper **III** describes a process given the name lyoconversion, where the lyoprotectant, mainly comprised of sucrose, was converted into bioactive exopolysaccharides. During lyoconversion, other bioactive compounds with no evident connection to sucrose were also produced, such as tryptamine,

indicating parallel use of stored substrates. Similarly, production of 3-hydroxypropionaldehyde, also known as the antimicrobial substance reuterin, in *L. reuteri* is reported to be best achieved in the stationary phase (although it is also affected by the initial cultivation) (Y. Sun et al. 2022). Another example is that amine accumulation and subsequent histamine production in *Lentilactobacillus hilgardii* only occurs in the stationary phase (Pessione et al. 2005).

These types of activities indicate a direction in which potentiation of different probiotics based on their enzymatic toolbox can be achieved. These activities can be used to develop synbiotic products where the strain together with the substrate is supplied to the intestine, where conversion begins. Allowing time for a substrate to be converted in the product is possibly an alternative way of achieving enriched probiotic products. However, conversion in the product probably has more implications than anticipated and thereby calls for further studies.

A suitable approach to identify metabolites formed during production would be to combine different types of chemical analyses. By using complementing methodologies including NMR, high-performance liquid chromatography (HPLC), and non-targeted GCMS in Paper **III**, it was possible to demonstrate that lyoconversion resulted in multiple interesting compounds being produced. A similar approach could be used to characterize novel strains, strain combinations, and production processes in general. Many of the compounds detected by HPLC or NMR could not be detected by GCMS, and *vice versa*. For example, acetic acid and 2,3-butanediol could not be detected with GCMS, due to their low boiling point, exemplifying the importance of using complementary methods. Note that the methods also have different detection limits. Many of the structures detected using GCMS in Paper **III** were present in low concentrations and could not be detected by NMR, at least not without fractionation and spiking of the samples with the metabolites, which was not possible within the timeframe of the study. The major strength of the combined methodologies approach is also a major drawback, namely finding a plethora of metabolites but with no absolute quantification, so knowledge about the meaning of these metabolites remains limited. However, the approach could be useful for identification of metabolic pathways. It could also act as a starting point for further evaluations using targeted metabolomics to decipher the metabolic

processes occurring during conversion and potentially during production of bioactive components in the intestine.

3.4 Summary

Effector compounds and structures secreted by probiotic bacteria could be important mediators of bioactive functions. MV have been studied intensively in recent decades and the findings, together with those presented in this thesis, indicate that they may be important contributing factors in the interactions with the host and with the microbiota. However, more research on the role of MV *in vivo* is required. Another finding is that the types of bioactive compounds produced are strain-specific, so unravelling the mechanisms by which they are produced can bring us one step closer to industrial production of enriched probiotic products.

4. Host interactions

Today, it is evident that the host is highly influenced by the complex microbial communities that reside within the gut. The phenotype (host) is a product of host and microbial gene expression and activities. This has led to the concept of holobionts, *i.e*., a host and its vast microbiota forming an ecological unit through symbiosis (Guerrero et al. 2013; Simon et al. 2019). The history of interactions between bacteria and humans started in the 1800s with the discovery of pathogens shown to cause disease, while it was not until the beginning of the 1900s that beneficial interactions were discovered (Dethlefsen et al. 2007). However, these bacteria have always been there, interacting and shaping life as we know it. A multitude of interactions occur between human cells and the gut microbiota, potentially beginning already in the womb and continuing throughout life.

Whether there is a fetal microbiome is a debated topic and it has been suggested that the fetus is not sterile (Walker et al. 2017). Many related studies have used different DNA-based methods, such as 16s RNA sequencing and qPCR, but a recent study highlighted the pursuit of a fetal microbiome as a cautionary example of hazards associated with sequence-based microbiome studies (Kennedy et al. 2023). While a fetal microbiome is unlikely, there is growing evidence that bioactive components, such as MV, short-chain fatty acids, and pathogen-associated molecular patterns from bacteria, can interact with the fetus by transplacental migration (Turunen et al. 2023; Surve et al. 2016; Thorburn et al. 2015; Miko et al. 2022). Interestingly, when bacterial RNA isolated from MV found in first-pass meconium was sequenced, it was found that 80% of total RNA belonged to the phyla *Firmicutes* (62%) and *Actinobacteria* (18%) (Turunen et al. 2023). The data also showed that most of the RNA isolated from the MV belonged to *Streptococcus* and *Staphylococcus*, but the origin of these

vesicles could not be determined. There was also a small portion of lactobacilli-derived MV (Turunen et al. 2023). How the fetus is influenced by the mother's microbiota through secretion of MV remains poorly studied. To the best of my knowledge, no studies to date have addressed transmission of probiotic derived MV from pregnant women receiving a probiotic intervention and potential presence in the fetal meconium. This might be an unexplored route of maternal microbiota influencing the fetus. Our research group has developed antibodies against the surface proteins 5'NT and glucansucrase of DSM 17938 and BG-R46 and their MV (data not shown). A potential future application of such specific antibodies could be to address this intriguing question.

4.1 Short chain fatty acids

Prenatal exposure to other bacterial components has been documented previously. As mentioned, short-chain fatty acids (SCFA) are among the major metabolites of bacterial metabolism and play an important role as an energy source for other bacteria. Studies on adults and pregnant mothers have shown that microbial composition and disrupted levels of serum SCFA may be associated with several metabolic diseases, including diabetes (type II and gestational), obesity, and arterial hypertension (Ziętek et al. 2021), as well as immune development and immunomodulation (Ziętek et al. 2021; Miko et al. 2022; Kimura et al. 2020). The signaling of maternal SCFA to the fetus is mediated by uteroplacental G-protein coupled receptors (GPCR) (Kimura et al. 2020; Gray et al. 2017; Miko et al. 2022). In mice, maternal SCFA has been shown to influence embryonic development through expression of the GPCRs GPR41 and GPR43 in the intestinal epithelium and sympathetic nerves (Kimura et al. 2020).

The most abundant SCFA is acetate (Nilsen et al. 2020), which is *e.g.*, an important substrate for butyrate producers in the microbiota (see section 2.3.2 of this thesis). Acetate also has direct effects on the host, *e.g.*, it has been demonstrated that fetal blood acetate levels increase in response to maternal high diet or acetate diet in mice (Thorburn et al. 2015). Acetate is not only an important substrate for microbial metabolism, but also an important mediator in fetal development. Reduced maternal levels of acetate have been associated with pre-eclamptic pregnancies and subsequent reduction of fetal regulatory T (T_{reg}) cells due to abnormalities in the thymus

(Hu et al. 2019). Strikingly, maternal supplementation with acetate can reverse the effect of pre-eclamptic pregnancy in germ-free mice by upregulating the autoimmune regulator known to stimulate T_{reg} generation (Hu et al. 2019). *Limosilactobacillus reuteri* produces acetate when supplied with electron acceptors and strain DSM 17938 affects T_{reg} deficiency and alleviates multiorgan inflammation in T_{reg}-deficient mice, ultimately prolonging their survival (He *et al.* 2017a). By producing anti-inflammatory adenosine via the enzyme 5'nucleotidase (5'NT, section 3.1.2), *L. reuteri* DSM 17938 and BG-R46 increase the abundance of regulatory CD73+CD8+ T cells through adenosine signaling (Liu *et al.* 2023). These findings indicate that *L. reuteri*-derived 5'NT and subsequent adenosine may be a central mediator in battling inflammation, and may have implications in the treatment of T_{reg}-associated immune disorders in humans (Liu *et al.* 2023). In addition, Liu *et al*. (2022) found that DSM 17938 supplementation increased the level of N-acetylated amino acids in newborn mice. Increased systemic acetate strongly impacts the acetylation of proteins (Moffett et al. 2020), and thus the findings by Liu *et al*. (2022) indicate that altered acetate levels could be another mechanism by which *L. reuteri* facilitates T_{reg} cells and ameliorates inflammatory diseases (Liu *et al.* 2022).

4.2 Metabolic diseases

Lean people are reported to have 30% higher acetate levels than obese people (K. F. Petersen et al. 2019). Additionally, supplementation with acetate in obese rats has been shown to normalize body weight and visceral fat mass (Olaniyi et al. 2021). Elevated levels of short-chain fatty acids (including acetate) have also been observed in obese people, with an increase of 20% compared with lean individuals (Schwiertz et al. 2010; K. N. Kim et al. 2019). Interestingly, it has been shown in mice that colonic acetate passes over the blood brain barrier, increasing hypothalamic acetate and thus also hypothalamic GABAergic neurotransmission, which is hypothesized to decrease appetite impulse (Frost et al. 2014). Thus, how acetate, obesity, and other metabolic disorders are associated remains somewhat contested, but the association is clear. Either way, maternal obesity has been shown to cause placental accumulation of macrophages, resulting in production of proinflammatory responses, and may have profound effects on the fetus

(Challier et al. 2008). For instance, the risk of fetal insulin resistance and diabetes mellitus development increases (Ziętek et al. 2021). Furthermore, immunological paralysis in peripheral monocytes in late gestation and at birth has been demonstrated (Sureshchandra et al. 2023). Whether probiotic supplementation with *e.g.*, *B. longum* BG-L47 and *L. reuteri* DSM 17938 has a role to play in battling maternal and fetal metabolic diseases remains unknown, but the outlook for probiotic interventions using carefully selected strains is promising. In terms of acetate production, the two strains complement each other, since *B. longum* BG-L47 has a broad fiber utilization capacity, resulting in acetate production, but is also able to supply *L. reuteri* DSM 17938 with a stimulatory molecule with electron-accepting properties, allowing it to produce acetate instead of ethanol (Paper **II**). In addition, *L. reuteri* DSM 17938 promotes acetylation of proteins in newborn mice, further indicating a role as an acetate-stimulating strain (Liu et al. 2022).

4.3 Inflammation associated diseases and disorders

Other bacterial metabolites have also been shown to have access to the fetus. For example, intrauterine administrations of lipopolysaccharides readily access the feto-placental unit and may impact the fetus (Brown et al. 2019). Bacterial cell wall peptidoglycan has been shown to pass over the murine placenta and elicit effects on fetal neurodevelopment (Humann et al. 2016). Another route by which the fetus can be affected by the maternal microbiota is through translocation of intestinal microbiota and microbiota-derived compounds into the bloodstream and placenta (Miko et al. 2022). It has also been postulated that maternal systemic low-grade inflammation could be an important signal that manifests as a dampened inflammatory response in the fetus. On the other hand, maternal allergic inflammation has been suggested to induce inflammation in the fetus, ultimately increasing the risk of allergy development, highlighting the complex interplay between maternal immune responses and the fetus (Thornton et al. 2010). It is known that inflammation and increased permeability promote bacterial translocation (Linares et al. 2021). In Paper **I**, MV derived from *L. reuteri* DSM 17938 and BG-R46 effectively abolished proinflammatory cytokine secretion in PBMC, and further strengthened the epithelial integrity. Thus, MV derived from those strains may elicit protective effects that transfer from the pregnant mother to

the fetus. Importantly, PBMC does not perfectly reflect the intestinal immune response, but can be useful in predicting immune responses in the intestine, even if the cell composition differs from that of intestinal tissue and lymph nodes innervating the intestine (Verhoeckx et al. 2015). A graphical illustration of the experimental procedures in the PBMC model used in this thesis is provided in Figure 5. Release of IL-1ß and IL-6, which are conventionally classified as proinflammatory cytokines, was measured in both Paper **I** and Paper **II**. However, the nature of inflammatory responses can vary widely (Thornton et al. 2010). IL-6 and IL-1ß have been associated with abundance of bifidobacteria in children and it has been suggested that *Bifidobacterium* colonization in early infancy impacts T cell maturation and may be important in infantile immune system maturation (Henrick et al. 2020; Rabe et al. 2020). Together with the complex biology of interleukins such as IL-6, the data support the notion that careful consideration is needed before aiming to suppress or block this immune mediator (Scheller et al. 2011). Inflammation and immune responses have previously been described as double-edged (Agrati et al. 2022). With contradictory data in terms of anti- and pro-inflammation, where conventional proinflammatory cytokines appear to serve as important signals shaping the immune system, it may be time for reassessment of proinflammatory and anti-inflammatory paradigms. A future research aim should be to better incorporate the complex greyscale of immune responses.

Interactions between probiotics and their derived metabolites with immune cells *in vitro* have been described by many (Forsberg et al. 2019; Grangette et al. 2005; van Hemert et al. 2010), and were demonstrated in Papers **I-III** in this thesis. However, while *in vitro* approaches represent a good way of evaluating probiotic strains, cell models lack the complexity of a host and do not show how probiotics and probiotic metabolites access the immune system. It has been demonstrated that *L. reuteri* induces immunoglobulin A (IgA) secretion by B cells located along the intestine in lymphoid organs known as Peyer's patches (Hao-Yu Liu et al. 2021). In that study, increased IgA production shifted the microbiota and conferred protection against dextran sulfate sodium-induced colitis and dysbiosis in mice. An intriguing task would be to evaluate whether probiotic-derived MV also interact by translocating to Peyer's patches, since it has already been demonstrated that MV derived from probiotic bacteria can interact with cells within Peyer's patches (Miyoshi et al. 2021;X. Wang et al. 2023). It is

possible that *L. reuteri*-derived MV interact in a similar way, as indicated by findings that IL-6 induction by MV from *Lactobacillus sakei* is crucial in augmented IgA production by B-cells (Miyoshi et al. 2021). In Paper **I**, IL-6 secretion was stimulated in naïve PBMC in response to *L. reuteri* DSM 17938- and BG-R46-derived MV.

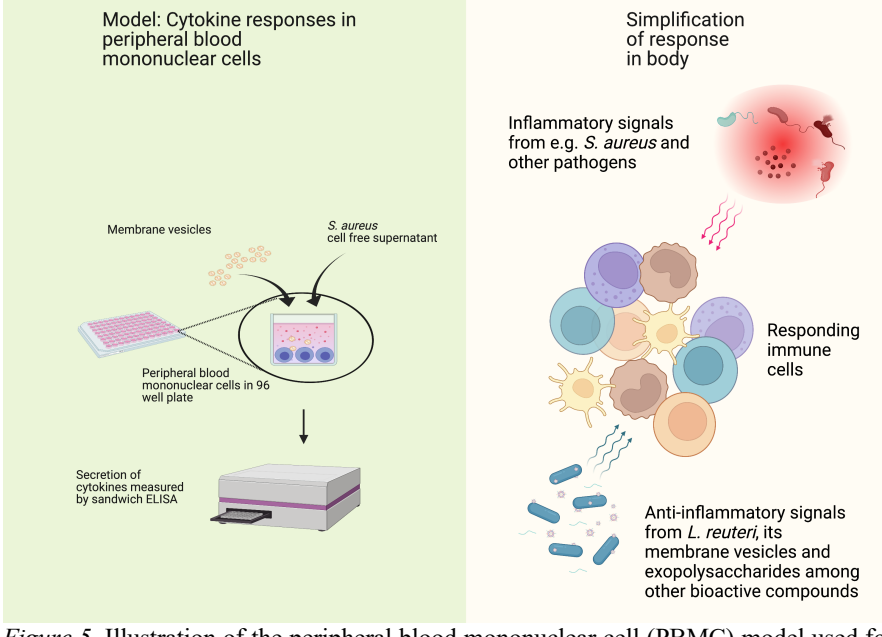


Figure 5. Illustration of the peripheral blood mononuclear cell (PBMC) model used for immune cell interactions. Naïve or *Staphylococcus aureus* stimulated PBMC were exposed to bacteria, membrane vesicles, lyoconverted samples, or exopolysaccharides and the cytokines released were measured with sandwich ELISA. The *S. aureus* sends inflammatory signals to the PBMC whereas the bacteria, membrane vesicles, lyoconverted samples, or exopolysaccharides send anti-inflammatory signals. This reflects an inflammatory site in the body to which PBMC migrate and help battle inflammation.

4.4 The importance of bacterial exposure early in life

Industrialized countries around the world have undergone a transition towards increased incidence of allergic and autoimmune diseases in recent decades, with a strong increase in hygiene measures being a major contributing factor. In parallel, the incidence of infectious diseases has declined (Bach 2002; Beasley and Committee 1998; Brooks *et al.* 2013;

Stiemsma *et al.* 2015). These observations support the 'hygiene hypothesis', which states that decreased exposure to microbes, especially early in life, results in immune dysregulation, with implications for disease development (Stiemsma et al. 2015; Okada et al. 2010; Brooks et al. 2013; Qin 2007; Bach 2002). It has also been demonstrated that children growing up on farms are less prone to develop allergies, which has been suggested to originate in immunotolerance development (Riedler et al. 2000). As mentioned, the fetus exits a sterile environment upon birth and is immediately exposed to the mother's vaginal and fecal microbiota, which inoculates the infant (Kennedy et al. 2023). Previous studies have shown that the infant gut microbiota of vaginally delivered infants resembles that of the mother's vaginal microbiota, whereas the microbiota of infants born by cesarean section is closer to that of the skin (Dominguez-Bello et al. 2010). More recent studies have demonstrated that the maternal fecal microbiota, rather than the vaginal microbiota, is transmitted during birth and sets the basis for the infant microbiota (Mitchell et al. 2020). It has been shown that the microbiota of infants born by cesarean section can be brought to resemble that of vaginally born infant microbiotas through maternal fecal transplantation (Korpela et al. 2020). The mode of delivery, feeding method, and antibiotic exposure can disturb the trajectory of the microbiota composition and development, *e.g.*, these factors have been associated with increased risk of allergy and asthma development and, together with maternal intrapartum prophylactic antibiotic treatment, can predispose infants to colonization by opportunistic pathogens (Shao et al. 2019). As mentioned, the most abundant bacteria in the infant microbiome is *Bifidobacterium*, which is found in low percentages in the vaginal microbiome, but has been shown to increase in pregnant mice feces in response to the maternal hormone progesterone during late pregnancy (Nuriel-Ohayon et al. 2019). Progesterone levels increase throughout pregnancy and peak in the third trimester, which indicates that the maternal fecal microbiome is altered in a preparatory way prior to birth, and ultimately initiates colonization of the infant intestine (Ouarabi et al. 2021). This type of preparatory process supports potential use of maternal supplementation of *Bifidobacterium* during pregnancy and continued infant use postnatally.

4.5 Impacts of feeding method

Feeding method, *i.e.*, breastfeeding or formula feeding, plays a major role in shaping the infant microbiota and in development of the immune system (Forbes et al. 2018). Establishment of the microbiota is dynamic during infancy, but stabilizes during childhood (Bäckhed et al. 2015). In early infancy, there is a timeframe that has been described as a 'window of opportunity' in which microbiota alterations induce long-term effects (van den Elsen et al. 2019). One study demonstrated that at four months of age there are distinct differences in the microbiota between exclusively breastfed and exclusively formula-fed infants, with that of breastfed infants being enriched in taxa commonly used as probiotics, including *Lactobacillus johnsonii/Lactobacillus gasseri*, *Lacticaseibacillus paracasei/Lacticaseibacillus casei* and *B. longum* (Bäckhed et al. 2015). Similar findings have been made by others (Bezirtzoglou et al. 2011; Pärnänen et al. 2022). Interestingly, microbial diversity appears to be higher in formula-fed infants (Ma et al. 2020; Pärnänen et al. 2022). Breastfeeding has multiple health benefits for the infant, including reduced risk of childhood obesity, severe respiratory illness, respiratory and gastrointestinal morbidity, and even sudden infant death syndrome (L. Wang et al. 2017; Duijts et al. 2010; Cushing et al. 1998; Hauck et al. 2011). Breastmilk contains a multitude of different bacteria, including bifidobacteria and lactobacilli (Notarbartolo et al. 2022), as well as components that benefit both the microbiota and the host (Soto *et al.* 2014; Łubiech and Twarużek 2020). It has been demonstrated that breastmilk-derived bifidobacteria colonize the infant gut microbiota (Martín et al. 2009). *Limosilactobacillus reuteri* and *B. longum* have both been isolated from human breast milk (Sinkiewicz and Ljunggren 2009; Gueimonde *et al.* 2007). It has also been demonstrated that human milk oligosaccharide (HMO)-degrading bifidobacteria produce aromatic lactic acids in the infant gut and that these compounds modulate the immune system by interacting with the aryl hydrocarbon receptor (AhR) and hydroxycarboxylic acid receptor 3 (HCA_3) (Laursen et al. 2021). Many lactobacilli are known to activate the AhR pathway by metabolizing dietary tryptophan and synthesizing ligands for the receptor (Huang et al. 2023). Ligand activation of AhR has many implications for health and disease, as reviewed elsewhere (Barroso et al. 2021). To summarize the aspects of relevance here, binding of an AhR ligand causes AhR to translocate to the nucleus, where it can have a multitude of

effects on transcription, including causing modifications of immune responses and alterations to intestinal barrier integrity (Stevens et al. 2009; Huang et al. 2023). Activation of AhR by lactobacilli has been shown to induce expression of IL-22, which provides colonization resistance to *Candida albicans* and suppresses mucosal inflammation in mice (Zelante et al. 2013). It also induces intestinal epithelial cell proliferation, aiding in maintenance of the intestinal barrier (Huang et al. 2023).

4.6 Intestinal barrier function

Strengthening of the intestinal barrier function is another way in which probiotics are believed to mediate their protective effects against multiple leakage and inflammation-associated disorders. A graphical illustration of the intestinal permeability model used in Paper **I** is provided in Figure 6. A leaky epithelium has been associated with many pathological conditions, including infantile colic, inflammatory bowel disease, irritable bowel syndrome, and autoimmune diseases (Daelemans *et al.* 2018; Camilleri 2019; Kinashi and Hase 2021). Increased permeability leads to inflammation of the intestinal mucosa, which causes immune cells to migrate, whereupon neutrophils and monocytes secrete calprotectin, which acts as an inflammatory signal of ongoing gastrointestinal inflammation (Daelemans et al. 2018; Pathirana et al. 2018).

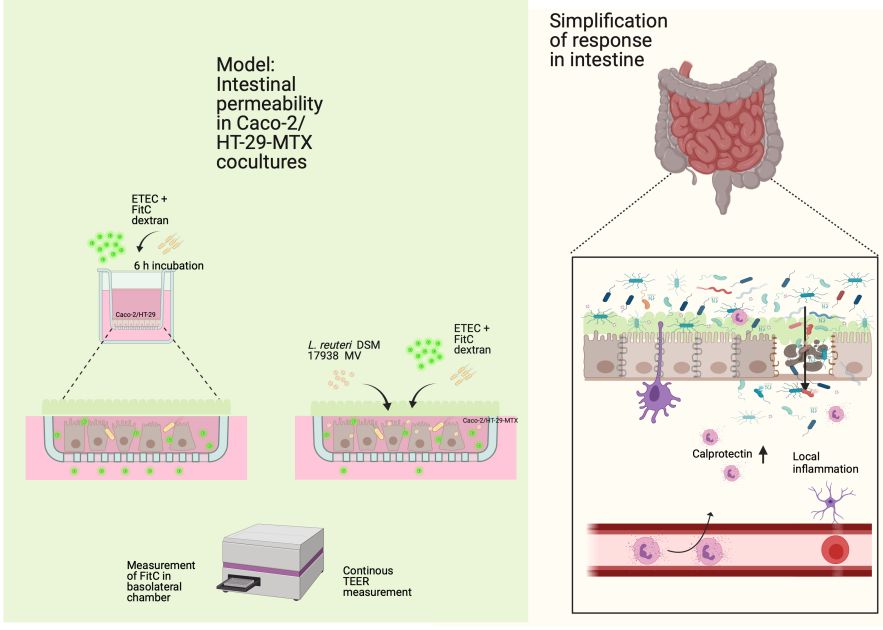


Figure 6. Graphical illustration of the intestinal permeability model used in Paper **I**. Caco-2/HT-29-MTX co-cultures were incubated with membrane vesicles prior to exposure to enterotoxigenic *Escherichia coli*, which caused rupture of the monolayer, but *Limosilactobacillus reuteri* DSM 17938- and *L. reuteri* BG-R46-derived membrane vesicles were able to protect against leakage. This reflects an inflamed intestine where potential pathogenic bacteria disturb the intestinal epithelium, reducing the tight junctions and triggering inflammation and an immune response.

However, calprotectin concentrations in infants are often elevated to levels comparable to those associated with irritable bowel disorder in adults (Li et al. 2015; Khaki-Khatibi et al. 2020). Elevated levels of calprotectin have also been found in infants with infantile colic compared with healthy infants, which may indicate increased neutrophilic infiltration (Rhoads et al. 2009). *Limosilactobacillus reuteri* DSM 17938 is well documented and multiple clinical trials have demonstrated its ability to ameliorate infantile colic (Sung et al. 2017). Interestingly, *L. reuteri* has been shown to reduce fecal calprotectin levels in colicky infants, indicating a reduction in intestinal inflammation (Savino et al. 2018). A negative association between *Bifidobacterium* abundance and fecal calprotectin has also been demonstrated (Henrick et al. 2019; Ray et al. 2022). Calprotectin levels appear to vary widely among infants, making comparisons and

generalizations difficult, but the fact that increased calprotectin levels occur in early infancy indicates that the epithelium is more permeable during infancy for a purpose. That purpose may be to facilitate training and maturation of the immune system, for instance by allowing transfer of maternal antibodies (Weström et al. 2020). This type of immune maturation facilitation probably relies on intricate balancing mechanisms, which may be disrupted during infantile colic. Furthermore, adequate regulation of such maturation-associated events possibly has long-term implications, since a recent study assessing an astounding 917,707 infants showed that infants suffering from infantile colic had an increased risk of developing irritable bowel syndrome after four years of age (J. H. Kim et al. 2022). Intestinal inflammatory diseases and disorders are associated with gastrointestinal pain, and it has been suggested that transient receptor potential vanilloid 1 (TRPV1) antagonists may play a role in alleviating these type of diseases (Csekő et al. 2019). Interestingly, this thesis showed that MV derived from *L. reuteri* DSM 17938 and BG-R46 not only protect against intestinal leakage induced by enterotoxigenic *E. coli* (Paper **I**), but also effectively antagonize the nociceptive receptor TRPV1 (Paper **I**), an effect boosted by *B. longum* BG-L47 (Paper **II**).

Intestinal permeability and barrier function are important features increasingly recognized as being of relevance in health and disease, and may be key in the complex interactions of probiotics and their health-promoting effects (Bischoff et al. 2014; Camilleri 2021). With the alarming rise of infections caused by antibiotic-resistant bacteria (https://www.who.int), alternative measures for battling infection are urgently required, and intestinal permeability has been shown to trigger a cascade of events that ultimately increases the risk of sepsis (Kumar et al. 2020). Interestingly, a synbiotic intervention has been shown to prevent neonatal sepsis among infants in rural India (Panigrahi et al. 2017). Probiotic or synbiotic supplementation could be an unconventional route of infection protection, potentially by strengthening the intestinal epithelium.

Intestinal permeability is also increased in diabetic patients (Bielka et al. 2022). A connection between diabetes and the microbiota has been established, and the microbiota has been shown to be modifiable and interconnected with dietary factors (Wu et al. 2020). Others have reported lower microbiota diversity in diabetic patients (Dedrick et al. 2020). However, a diet promoting butyrate and acetate has been shown to increase

intestinal integrity and reduce diabetogenic cytokines in non-obese diabetic mice (Mariño et al. 2017). These observations may indicate that ecological niches are lost in diabetic patients. It has been reported that children with type 1 diabetes have lower abundance of *Bifidobacterium*, while butyrate-producing bacteria have been shown to be reduced in prediabetes and type 2 diabetes (Lakshmanan et al. 2021; Wu et al. 2020). These findings suggest that acetate-producing bacteria, and subsequently butyrate-producing bacteria, are negatively affected during metabolic diseases such as diabetes. Thus, there may be unexplored potential in promoting ecological restoration, for instance by a high-fiber diet and probiotic supplementation.

5. Conclusions and future perspectives

Lactobacilli and bifidobacteria are important members of the human microbiota and *Limosilactobacillus reuteri* and *Bifidobacterium longum* are species with promising effects on the host when used as probiotics. *Limosilactobacillus reuteri* DSM 17938 is one of the most well-studied probiotic strains and is used *e.g.*, to treat infantile colic. The main conclusions and future perspectives from the work in this thesis are:

- *Limosilactobacillus reuteri* DSM 17938 and *L. reuteri* BG-R46 produce potent membrane vesicles (MV) which elicit many effects of bacterial cells believed to be important for their clinical effects.
- *Bifidobacterium longum* BG-L47 possesses desirable attributes and is safe to consume as a probiotic.
- *Bifidobacterium longum* BG-L47 can effectively boost growth of *L. reuteri* DSM 17938, as well as bioactive attributes of the derived MV.
- Production of probiotics at industrial scale differs from that at laboratory scale, but lyoconversion is a possible intervention window where bioactive structures can be produced.
- *Limosilactobacillus reuteri* DSM 17938 effectively converts sucrose to an exopolysaccharide (glucan) and other bioactive structures.
- MV derived from *L. reuteri* strains DSM 17938 and BG-R46 can abolish proinflammatory immune responses triggered by *Staphylococcus aureus* in peripheral blood mononuclear cells, ameliorate leakage induced by enterotoxigenic *Escherichia coli* in Caco-2/HT-29-MTX co-culture, and further antagonize the nociceptive receptor TRPV1 in a dorsal root ganglion cell model. These effects demonstrate that MV are potent effector structures

produced by bacteria that can interact with different cell types. How *L. reuteri*-derived MV act *in vivo* requires further investigation, although it is tempting to speculate that the main interaction site is Peyer's patches.

- MV are affected by cultivation approach and presence of other bacteria, which indicates that those produced *in vivo* may differ substantially from those produced under laboratory conditions.
- Variations in membrane vesicle composition and number can be studied using NMR with complementary nanoparticle tracking analysis. Using DNA and RNA sequencing to better characterize the contents of MV would help in understanding other functions delivered or affected by MV. How MV derived from probiotic species interact with other bacteria also requires further investigation. Finally, the fact that MV and bacterial cells elicit similar effects *in vitro* raises the question of whether the bacteria need to be live when administered, and thus a head-to-head study addressing effect of probiotics versus postbiotics is warranted.
- *Bifidobacterium* and other primary degraders of the human intestine are important in construction of ecological niches. *Limosilactobacillus reuteri* is able to grow in a simulated intestinal medium when supplied with an electron acceptor. Computational models combined with experimental data could potentially be used to identify the potential electron acceptors produced by *B. longum*. Acetate is produced by bifidobacteria, but also by *L. reuteri* and other lactobacilli when supplied with an electron acceptor. Acetate has many effects on the host and serves as a substrate for butyrate producers. Thus, probiotics should perhaps be accompanied by recommendations on a high-fiber diet that promotes acetate production, or fiber should even be delivered as a synbiotic to reach the full potential. Studies addressing the role of acetate in probiotic clinical studies are needed, as are clinical trials where specific species with complementary tasks are evaluated together.

- *Limosilactobacillus reuteri* BG-R46 demonstrated increased potency in some preclinical models and should be evaluated in clinical trials. The strain should be evaluated alone or in combination

with DSM 17938 to determine whether it can ameliorate infantile colic in a more effective manner.

- *Bifidobacterium longum* BG-L47 is a promising and safe strain that is compatible with *L. reuteri* DSM 17938. Given its ability to metabolize HMOs and plant-derived fibers, *B. longum* BG-L47 may hold potential as a probiotic strain suitable from infancy to adulthood. Future clinical trials are needed to elucidate its clinical effects.
- Further studies are also needed on lyoconversion, as multiple questions remain unaddressed, including: (*i*) whether the detrimental effects on freeze-drying survival can be avoided or countered; (*ii*) whether lyoconversion can be used as a tool to generate more bioactive probiotics or is simply a side-effect of production that should be avoided; (*iii*) whether lyoconversion is an unexplored window for production of bioactive compounds, and whether other effector structures be produced in a similar fashion simply by supplying the substrate; and (*iv*) whether conversion systems can be induced, causing the bacteria to be more active in the intestine.

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Popular science summary

Lactobacilli and bifidobacteria are types of bacteria with profound effects on human health. They can act as probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The species *Limosilactobacillus reuteri* (*L. reuteri*) most likely evolved with humans over a long time, but following recent lifestyle-associated changes in Westernized populations it has largely vanished from the human intestine. In the immense landscape of the intestinal microbiota, *i.e.*, the bacteria that reside within the gut, complex cooperation's and competitions take place, ultimately creating an ecological system. Within this system, different bacteria have different functions which, when combined, allow the bacteria to survive and affect the host.

The intestine has a continuous supply of nutrients that originate in the diet. Human gut cells can use some of these components as energy, but much of the fraction referred to as dietary fiber is actually undegradable by humans. Fibers are present in essentially all dietary components consumed throughout life, including breast milk and plant-derived foods, and fiber-degrading bacteria are key members of the human gut microbiota. Bifidobacteria and lactobacilli are part of the human microbiota and bifidobacteria are one of the main fiber-degrading bacteria. Two strains of *L. reuteri* (named DSM 17938 and BG-R46) and three strains of *Bifidobacterium longum* (*B. longum*) (named BG-L47, BG-L48, and BB536) were studied in this thesis.

Paper **I** evaluated the release of small structures that can act at a distance from the bacteria of origin, called membrane vesicles, released by the two strains of *L. reuteri*. The results showed that membrane vesicles, are secreted in high amounts from the bacterial cells, where they appear to bud from the surface and are subsequently released to the surroundings. Further analyses

demonstrated that membrane vesicles have different physiological properties, such as size, number, and enzymatic activity, depending on the strain that releases them. Even larger differences were seen upon comparing the vesicles obtained from *L. reuteri* with those from another well-studied probiotic lactobacilli called *Lacticaseibacillus rhamnosus* GG (LGG). The hypothesis that membrane vesicles interact with human cells and mediate beneficial effects was evaluated in several human and mammal cell models including: (*i*) intestinal cells exposed to stress in the form of a bacteria causing disease and rupture of the layer of cells; (*ii*) cells of the human immune system, which were either unexposed to bacteria prior to encountering the membrane vesicles, or exposed to disease-causing bacteria that often trigger inflammatory responses in these cells; and (*iii*) cells derived from the rat nervous system (neurons), which are responsible for sending pain signals through a specific target on the cell (a receptor called TRPV1). The results showed that the membrane vesicles were able to produce the same beneficial effects observed in response to *L. reuteri* bacterial cells. In short, leakage from the intestinal cells was reduced, the inflammatory response caused by disease-causing bacteria was alleviated, and pain signaling was reduced. Another important finding was that *L. reuteri* BG-R46 had slightly stronger enzymatic activity and stronger effects in several models. These models in part reflect how infantile colic is believed to occur in infants, and the results provide a potential explanation as to why *L. reuteri* DSM 17938 can relieve infantile colic in babies. Comparisons of the surface of the bacteria with the surface of the membrane vesicles revealed that the overlap was only around 25 %, indicating that the contents of the membrane vesicles are selective and not random.

In Paper **II**, we looked for novel *B. longum* strains able to boost *L. reuteri* DSM 17938, and thus perform a supportive role. Three strains of *B. longum* were compared in terms of their *L. reuteri* DSM 17938 boosting capacity and basic probiotic characteristics *i.e.*, their ability to grow on different sugars and fiber, and to survive the stressful environment throughout the digestive tract. The results showed that all three *B. longum* strains boosted *L. reuteri* DSM 17938 growth in a medium resembling intestinal fluid. The results also showed that *B. longum* BG-L47 could grow on many different sugars and fiber types and was more tolerant to low pH (reflecting the gastric juice) and bile (reflecting the bile acids released in the intestine). Further, *B. longum* strain BG-L47 was able to bind to the mucus that lines the intestinal tract.

Such binding is believed to be a desirable trait of probiotics, as it allows the bacteria to get within interaction range of human cells. Based on these findings, *B. longum* BG-L47 was selected as a candidate probiotic and compared with another well-studied strain of *B. longum* called BB536. To test the hypothesis that membrane vesicles of *L. reuteri* DSM 17938 are boosted by *B. longum*, the bacteria were cultured together, the membrane vesicles were extracted, and their effects were investigated in similar cell models to models (i)-(iii) mentioned above. The results demonstrated that *B. longum* strains BG-L47 and BB536 both increased the potency of *L. reuteri* DSM 17938 membrane vesicles, but that the boosting effects differed between the strains. Both strains increased stimulation of immune cells. Interestingly, strain BG-L47 appeared to further increase the enzymatic activity and pain-reducing capacity of the membrane vesicles, which was an effect unique for *B. longum* BG-L47 as a booster. This warranted further investigation of *B. longum* BG-L47 as a stand-alone probiotic strain. It was therefore evaluated in a randomized clinical study on humans, where it was demonstrated to be safe and well-tolerated by the participants. Supplementation with strain BG-L47 did not cause any changes in the dominant microbiota, although some differences were seen in terms of increased and decreased abundance of certain bacteria. Thus, the results demonstrated that *B. longum* BG-L47 is a promising strain for boosting the activity of *L. reuteri* DSM 17938 and is safe for human consumption. The strain is a potent sugar and fiber degrader and tolerates the stressful environment of the human digestive tract, making it a strong candidate strain for further evaluations in clinical studies.

It is known that there are several phases during industrial production of probiotics where the bacteria may be held for hours before the progress to the next process step often referred to as holding time. A third study in this thesis, paper **III**, evaluated whether holding time affects the composition of the end-product or not. After probiotic bacteria are cultivated in industrial production, they are often freeze-dried. Before freeze-drying, the bacteria are generally provided with some protection against these harsh conditions. This often involves placing the bacteria in sugar solution, where the sugar used needs to be non-reducing in order to attain higher survival after the drying. Sucrose is commonly used and genetic studies on *L. reuteri* DSM 17938 have revealed that the strain possesses an enzyme that can convert sucrose into longer chains of sugar called exopolysaccharides. A novel finding in this

thesis was that conversion of sucrose into exopolysaccharides (a process named lyoconversion) can occur in the conditions applied through the probiotic production process. The results showed that sucrose was converted to a great extent and confirmed that an exopolysaccharide was formed. The exopolysaccharide obtained was characterized using nuclear magnetic resonance (NMR) that revealed that many other products, apart from the exopolysaccharide, were also formed. Lyoconversion was also shown to have a severe impact on bacterial cells, which were much less likely to survive the freeze-drying process if the sucrose had been converted. Analyses of other metabolites formed during the process showed that they included many compounds with potential biological relevance. The overall results indicated negative effects on the fitness of the probiotic bacteria, but led to the hypothesis that the biological activity of the finished product could be increased by allowing lyoconversion. This hypothesis was supported by the data, with lyoconverted samples having greater effects on immune cells than unconverted samples. The data confirmed that part of this effect was due to presence of the exopolysaccharide, which also stimulated the immune cells.

In conclusion, evaluations using cell models indicated that *L. reuteri* DSM 17938 releases potent membrane vesicles that may have broad effects on relevant target cells in the human body. Future studies are needed to reveal if these effects occur in a human host. *Bifidobacterium longum* BG-L47 is a strain with promising probiotic characteristics, as it stimulates *L. reuteri* DSM 17938 and is safe for human consumption. Lyoconversion effectively converts sucrose into exopolysaccharide during industry-relevant holding times, which may have implications for the probiotic efficiency of the finished product, but this appears to come at the cost of lower survival of the bacteria.

Populärvetenskaplig sammanfattning

Laktobaciller och bifidobakterier är två typer av bakterier som har djupgående effekter på människors hälsa. De klassificeras inom definitionen av probiotika som är levande mikroorganismer som när de konsumeras i tillräckliga mängder ger en hälsofördel för värden. *Limosilactobacillus reuteri*, (*L. reuteri*) är en bakterie som sannolikt har utvecklats tillsammans med människor under lång tid, men som påverkats av livsstilsförändringarna i det västerländska samhället och försvunnit från den mänskliga tarmen. I den enorma tarmmikrobiotan, det vill säga bakterierna som finns i våra tarmar, pågår komplexa samarbeten och konkurrens som formar ett ekologiskt system. Inom ramen för detta ekosystem har olika bakterier särskilda funktioner och de kombinerade funktionerna gör att de olika bakterierna kan överleva och påverka värden (människan). Tarmen har en kontinuerlig tillförsel av näringsämnen från maten som vi äter och våra mänskliga celler kan använda vissa av dessa komponenter som energi, medan andra inte kan användas. Många av de vi kallar kostfibrer kan vi inte själva bryta ned. Dessa fibrer finns i stort sett i all vår mat under hela livet, inklusive i bröstmjölk och växtbaserad mat. Det är för detta ändamål som fibernedbrytande bakterier är nyckelmedlemmar i vår mikrobiota, och bland dessa fibernedbrytande bakterier återfinns bifidobakterier. I denna avhandling studerades några stammar av *L. reuteri* (benämnda DSM 17938 och BG-R46) och av *Bifidobacterium longum* (*B. longum*) (benämnda BG-L47, BG-L48 och BB536). I den första artikeln (artikel **I**) utvärderade vi hur de två stammarna av *L. reuteri* kan frigöra små strukturer som likt drönare kan verka långt från ursprungsbakterien Dessa kallas membranvesiklar. Vi visade att dessa membranvesiklar utsöndras i stora mängder från bakteriecellerna, att de verkar knoppa av ytan och sedan släppas ut i omgivningen. Resultaten visade att fysiologiska egenskaper såsom storlek,

antal och enzymatisk aktivitet är olika beroende på vilken bakteriestam de kommer ifrån. Ännu större skillnader erhölls när vi jämförde dem med en annan välstuderad probiotisk laktobacill som heter *Lacticaseibacillus rhamnosus* GG (LGG).

Vi antog att membranvesiklarna kunde interagera med mänskliga celler och förmedla fördelaktiga effekter. Detta utvärderade vi i flera cellmodeller inklusive: i) mänskliga tarmceller exponerade för stress i form av en bakterie som orsakar sjukdom och bryter det yttersta skiktet av celler närmast tarmens innehåll , ii) celler i det mänskliga immunsystemet som antingen inte var exponerade för bakterier före interaktion med membranvesiklarna, eller som exponeras för sjukdomsframkallande bakterier som ofta utlöser inflammatoriska svar i dessa celler parallellt med membranvesiklarna, iii) celler som härrör från råttans nervsystem (neuroner) som är ansvariga för att skicka smärtsignaler till cellen (via en receptor som kallas TRPV1). Resultaten visade att membranvesiklarna kunde ge samma fördelaktiga effekter som hade observerats som svar på *L. reuteri* bakterieceller. Kort sagt minskade läckaget av tarmcellerna och det inflammatoriska svaret orsakat av sjukdomsframkallande bakterier lindrades samt smärtsignaleringen minskade. En annan observation var att BG-R46 hade något högre enzymatisk aktivitet och starkare effekter i flera modeller. Dessa modeller återspeglar delvis hur vi tror att spädbarnskolik orsakas, och vi föreslår att dessa resultat ger en möjlig förklaring till hur DSM 17938 kan lindra just spädbarnskolik. I artikel **I** jämförde vi också proteiner på bakteriernas yta med proteiner på membranvesiklarnas yta och överlappet visade sig var endast cirka 25 %, vilket tyder på att ett urval av innehållet i och på membranvesiklarna sker.

I artikel **II** sökte vi efter nya *B. longum*-stammar som kunde förbättra de probiotiska effekterna av DSM 17938 och därmed utgöra en stödjande partnerstam. Vi jämförde flera stammar av *B. longum* i förhållande till deras DSM 17938-stimulerande kapacitet samt grundläggande probiotiska egenskaper. Probiotiska egenskaper syftar på deras förmåga att växa på olika sockerarter och fibrer samt överleva den stressiga miljön i hela matsmältningskanalen. Vi upptäckte att alla tre testade *B. longum* ökade tillväxten av DSM 17938 i ett medium som liknade tarmens vätskeinnehåll. Vi visade också att BG-L47 kunde växa på många olika sockerarter och fibrer, var mer tolerant mot lågt pH (som speglar magsaften) och gallsyror. BG-L47 vidhäftade till slem som kantar tarmkanalen vilket tros vara en

önskvärd egenskap hos probiotiska bakterier eftersom det gör att de finns inom räckhåll för interaktioner med mänskliga celler. Vi valde BG-L47 som en kandidatstam baserat på dessa fynd och valde att jämföra den med en annan välstuderad stam av *B. longum* kallad BB536. Vår nästa hypotes var att membranvesiklarna i DSM 17938 också stimulerades av *B. longum*. Därför odlade vi bakterierna tillsammans och extraherade membranvesiklarna och undersökte deras effekter i liknande modeller som beskrivs i artikel **I**. Resultaten visade att både BG-L47 och BB536 ökade effekterna av DSM 17938-membranvesiklarna men att egenskaperna som förstärktes skilde sig åt mellan de två stammarna. Båda stammarna ökade stimuleringen av immunceller. Intressant nog verkade BG-L47 ytterligare öka den enzymatiska aktiviteten samt den smärtreducerande kapaciteten hos membranvesiklarna vilket var en effekt som var unik för BG-L47 som stöttande stam. Det här ledde till ytterligare undersökningar av BG-L47 som en fristående probiotisk stam, och den utvärderades i en randomiserad klinisk studie på människor. BG-L47 visades vara säker och väl tolererad av deltagarna. Tillskottet av BG-L47 gav inga förändringar av den dominanta mikrobiotan även om vissa skillnader kunde ses i form av ökad eller minskad förekomst av vissa bakterier. Sammanfattningsvis visade artikel **II** att BG-L47 är en lovande stam som kan öka aktiviteten hos *L. reuteri* DSM 17938, och är säker vid mänsklig konsumtion. Stammen är en potent socker- och fibernedbrytare och tolererar den stressiga miljön i det mänskliga matsmältningssystemet vilket gör den till en kandidatstam för ytterligare utvärderingar i kliniska studier.

I artikel **III** utvärderade vi om en viss fas av industriell produktion av probiotika som kallas hålltid påverkar innehållet i den färdiga produkten. Det är känt att under produktion av probiotika finns det flera faser där bakterierna kan stå still i timmar vilket då kallas hålltid. Efter odling av bakterierna ska de frystorkas och mellan dessa två steg är den allmänna praxisen att ge bakterierna ett visst skydd mot den hårda hanteringen som frystorkning innebär. Detta görs ofta genom att sätta bakterierna i en sockerlösning där sockret behöver vara icke-reducerande, vilket i detta fall kan översättas till att det är stabilt i vatten, för att ge en högre överlevnad efter frystorkningen. Sackaros är vanligt förekommande i dessa sammanhang och tidigare kunskaper om genetiken hos DSM 17938 har visat att stammen har ett enzym som kan omvandla sackaros till längre sockerkedjor som kallas exopolysackarider (EPS). Det är dock inte allmänt känt att denna typ av

omvandling av sackaros till EPS (i artikeln kallad lyokonvertering) sker under förhållanden som tillämpas under produktionsprocessen. Resultaten visade att sackaros omvandlades i stor utsträckning och att en EPS bildades enligt hypotesen. EPS karakteriserades med hjälp av kärnmagnetisk resonans (NMR). Vi kunde då också observera att det utöver EPS också bildades många andra produkter vid omvandlingen. Lyokonverteringen visade sig också påverka bakteriecellerna negativt, då de var mycket mindre benägna att överleva frystorkningen om sackarosen hade omvandlats. Därefter ville vi reda ut vilka andra metaboliter som hade bildats under processen och resultaten visade att många föreningar med potentiell biologisk relevans också hade bildats. Hittills hade effekterna framstått som negativa vad gäller de probiotiska bakteriernas fysiologiska egenskaper. Vår hypotes var dock också att lyokonvertering kunde öka den biologiska aktiviteten hos den färdiga produkten. Denna hypotes förefaller vara sann då de konverterade proverna hade större effekter på extraherade immunceller än okonverterade prover. Vi verifierade också att en del av denna effekt berodde på EPS som också stimulerade immuncellerna.

Sammanfattningsvis frisätter DSM 17938 potenta membranvesiklar med effekter på relevanta målceller i människokroppen vilket utvärderades i olika cellmodeller (artikel **I**). Framtida studier kan undersöka om dessa effekter också förekommer i människor. BG-L47 är en stam av *B. longum* som har lovande probiotiska egenskaper, stimulerar DSM 17938 och är säker för mänsklig konsumtion (artikel **II**). Lyokonvertering omvandlar effektivt sackaros till EPS under industrirelevanta förhållanden vilket kan ha fördelaktiga konsekvenser för den probiotiska effektiviteten hos den färdiga produkten. Det tycks dock komma på bekostnad av bakteriernas överlevnad (artikel **III**).

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Extracellular membrane vesicles from *Limosilactobacillus reuteri* strengthen the intestinal epithelial integrity, modulate cytokine responses and antagonize activation of TRPV1

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Bacterial extracellular membrane vesicles (MV) are potent mediators of microbe-host signals, and they are not only important in host-pathogen interactions but also for the interactions between mutualistic bacteria and their hosts. Studies of MV derived from probiotics could enhance the understanding of these universal signal entities, and here we have studied MV derived from *Limosilactobacillus reuteri* DSM 17938 and BG-R46. The production of MV increased with cultivation time and after oxygen stress. Mass spectrometry-based proteomics analyses revealed that the MV carried a large number of bacterial cell surface proteins, several predicted to be involved in host-bacteria interactions. A 5′-nucleotidase, which catalyze the conversion of AMP into the signal molecule adenosine, was one of these and analysis of enzymatic activity showed that *L. reuteri* BG-R46 derived MV exhibited the highest activity. We also detected the TLR2 activator lipoteichoic acid on the MV. In models for host interactions, we first observed that *L. reuteri* MV were internalized by Caco-2/HT29-MTX epithelial cells, and in a dose-dependent manner decreased the leakage caused by enterotoxigenic *Escherichia coli* by up to 65%. Furthermore, the MV upregulated IL-1β and IL-6 from peripheral blood mononuclear cells (PBMC), but also dampened IFN-γ and TNF-α responses in PBMC challenged with *Staphylococcus aureus*. Finally, we showed that MV from the *L. reuteri* strains have an antagonistic effect on the pain receptor transient receptor potential vanilloid 1 in a model with primary dorsal root ganglion cells from rats. In summary, we have shown that these mobile nanometer scale MV reproduce several biological effects of *L. reuteri* cells and that the production parameters and selection of strain have an impact

on the activity of the MV. This could potentially provide key information for development of innovative and more efficient probiotic products.

KEYWORDS

extracellular membrane vesicles, *Limosilactobacillus reuteri*, microbe-host interaction, immune response, epithelial cells integrity, TRPV1 pain receptor, proteomics, probiotics

Introduction

In recent years, there has been an increased interest in extracellular membrane vesicles (MV), which are abundant in nature and released in an evolutionally conserved manner by different types of organisms (Raposo and Stoorvogel, 2013; van Niel et al., 2018). Additionally, synthetic lipid nanoparticles with similarities to MV (Skotland et al., 2020) have been extensively used as vectors for delivery for vaccines, most recently in multiple vaccine candidates for SARS-CoV-2 (Lu et al., 2020; McKay et al., 2020). Bacteria-derived MV can affect diverse biological processes and have emerged as potentially important mediators of pathogen-host interactions (Jan, 2017) and bacteria to bacteria interactions (Caruana and Walper, 2020). It has also been demonstrated that MV represent a strategy for communication between beneficial bacteria and intestinal epithelial cells (Canas et al., 2016). Most of the research has addressed the functions of MV from Gram-negative bacteria and mammalian cells. Gram-positive bacteria, to which most probiotics belong, were initially believed to not produce MV, but during the last decades numerous studies have demonstrated the opposite (Lee et al., 2009; Brown et al., 2015). For instance, it has been described that probiotic bacteria-derived MV could inhibit HIV-1 infection of human tissues (Nahui Palomino et al., 2019), limit the growth of hepatic cancer cells (Behzadi et al., 2017), regulate brain function (Haas-Neill and Forsythe, 2020), and modulate inflammatory responses (Kim et al., 2018; Mata Forsberg et al., 2019; Yang et al., 2019; Choi et al., 2020).

Limosilactobacillus reuteri DSM 17938 is one of the most well studied probiotic strains and has been under intense investigation for many years (Walter et al., 2011; Mu et al., 2018). The best-described clinical effect is amelioration of infantile colic, which has been reported in a number of clinical trials (Savino et al., 2010; Szajewska et al., 2012) and confirmed in several meta-analyses (Gutierrez-Castrellon et al., 2017; Sung et al., 2018). The mechanism behind these effects is likely of a complex nature and is, like the etiology of colic, far from being completely understood (Zeevenhooven et al., 2018). However, strain DSM 17938 has in preclinical investigations shown a number of effects that are believed to be important for the relief of colic: several studies have demonstrated its ability to ameliorate inflammation (Hoang et al., 2018; Karimi et al., 2018) and improve the intestinal epithelial barrier function (Karimi et al., 2018); it has also been shown that the strain reduces signaling from the transient receptor potential vanilloid 1 (TRPV1) channel which is a major nociceptive receptor in the intestine (Perez-Burgos et al., 2015); and finally, DSM 17938 has the ability to modulate intestinal motility in an *ex vivo* mouse model (Wu et al., 2013). Interestingly, it has been described that *L. reuteri* DSM 17938 produce MV (Grande et al., 2017) and that those mediate a similar effect on gut motility (West et al., 2020) and possess an immune modulatory activity (Mata Forsberg et al., 2019).

To further increase the knowledge about MV from *L. reuteri*, we have investigated the physicochemical and biological composition of MV derived from two *L. reuteri* strains, and factors affecting MV production. DSM 17938 is a well-described strain with proven probiotic efficacy, and BG-R46 is a related and novel strain of *L. reuteri* with improved *in vitro* properties, making the comparison between the two strains interesting to pursue. We have also investigated the effects of the MV in three types of cell models: intestinal permeability in a Caco-2/HT29-MTX epithelial cell model; immune modulatory effects in a peripheral blood mononuclear cells (PBMC) model; and their ability to dampen activation of TRPV1 in primary rat dorsal root ganglion cells (rDRGs). This study both provides basic knowledge of *L. reuteri* derived MV as well as teaches us about their potential role as effectors involved in probiotic mechanisms, with a focus on infantile colic.

Materials and methods

Bacterial strains

Two strains of *L. reuteri* subsp. *kinnaridis* have been used in the study. The first, *L. reuteri* DSM 17938, is a well-studied and widely used strain (Rosander et al., 2008; Li et al., 2021). The second strain *L. reuteri* BG-R46 (also designated DSM 32846) has been obtained after selective breeding of DSM 17938. This strain was cultivated overnight in Man–Rogosa–Sharpe (MRS) broth and thereafter incubated in MRS broth containing 0.5% porcine bile (B8631, Sigma Aldrich) at 37°C for 90 min. The suspension was diluted and plated on MRS agar plates, which were incubated anaerobically at 37°C for 16 h. Among the selected colonies, one isolate was found to have a stable phenotype with significantly smaller colony size and increased secreted 5′ nucleotidase activity compared to DSM 17938. The pure strain was named BG-R46. Both *L. reuteri* DSM 17938 and BG-R46 have been used with permission of BioGaia AB, Stockholm, Sweden.

In addition, *Lacticaseibacillus rhamnosus* strain GG (ATCC 53103) was used as a comparison strain in some of the experiments.

Cultivation of bacteria, isolation, and basic characterization of extracellular MV

The strains were grown in MRS medium (Oxoid) under different conditions: at 37°C without/with agitation (120 rpm rotation from the beginning of cultivation; the volume size of flask/the volume of culture = 5:1), harvested after 24 or 48 h, separated from the culture broth by centrifugation at 5,000 × *g* for 10 min at 4°C, followed by centrifugation at 10,000 × *g* for 10 min at 4°C, after which any residual cells were removed from the supernatants by filtration using a 0.45 μm pore filter. Supernatants were concentrated using Amicon 100 kDa MWCO (molecular weight cutoff) filter columns. Thereafter the supernatants were centrifuged in a Beckman Coulter Optima L-80XP ultracentrifuge (Beckman Coulter, United States) at 118,000 × *g* at 4°C for 3 h. The supernatants were discarded, the pellets resuspended in PBS buffer and thereafter ultra-centrifuged for a second time (118,000 × *g* at 4°C for 3 h). The pellets were finally suspended in PBS, aliquoted and stored at-70°C. Meanwhile, *L. rhamnosus* GG (LGG) was cultivated in MRS at 37°C without shaking for 24 h. LGG derived MV were used as controls.

The protein and nucleic acid contents of the MV preparations were quantified by using Qubit protein, double stranded DNA and RNA assay kits (Invitrogen) according to the manufacturer's instructions. All measurements were carried out as three independent experiments.

L. reuteri DSM 17938 and BG-R46 bacterial growth was determined by optical density (OD_{600}) and colony forming unit (CFU) measurements during cultivation. *L. reuteri* DSM 17938 and BG-R46 were grown in the MRS medium (Oxoid) at 37°C without agitation for 48 h. *L. reuteri* DSM 17938 was also grown in the MRS medium (Oxoid) under the O2 stress condition (120 rpm rotation from the beginning of cultivation; the volume size of the flask/ the volume of the culture=5:1) for 48 h. The optical densities were measured by using a spectrophotometer. CFU were measured after 0, 3, 5, 24 and 48 h cultivation time respectively for each culture. MRS agar (Oxoid) plates incubated anaerobically at 37°C for 48 h were used for CFU determination. The results were expressed as log CFU/ml.

Morphological characterization of MV by transmission electron microscope (TEM) and scanning electron microscope (SEM)

Ten μl of the MV suspensions were added on a carbon coated grid (2 mm) after dilution with PBS. The grid was maintained at room temperature for 5 min and after excessive liquid was absorbed by filter paper, the MV were negatively stained by 2% Uranyl acetate for 3 min. The MV were rinsed by PBS and then aired before observed and photographed by TEM (H-8100, Hitachi, Tokyo, Japan) at 80–120 kV.

Limosilactobacillus reuteri bacterial cells were centrifuged for 20 min at 4000 x *g* at 4°C, washed twice with PBS (pH 7.4), loaded on poly-L-Lysine coated silica wafer substrate, and fixed for 1–2 h at room temperature in the dark with 1% v/v osmium tetroxide in 0.1 M PIPES buffer. After washing three times in PBS buffer, the samples were dehydrated through an ethanol gradient (30, 50, 70, 85, 95, 100%; 15 min each) and treatment with hexamethyldisilazane. Finally, they were sputter coated with gold and photographed by using SEM (Zeiss Gemini 450 II, Zeiss, Oberkochen, Germany).

Proteomics

Biological triplicates of MV from DSM 17938 and BG-R46 were prepared, pooled and split into three technical replicates. Each MV fraction was isolated from 200 ml cultivations and the final volume were 200 μl per MV fraction. The surface proteome (surfaceome) of the MV fractions were analyzed by the lipid-based protein immobilization (LPI) methodology (Karlsson et al., 2012). Fifty μl was used to fill one LPI channel, and three different channels were used per MV strain. Channel 1–3 consisted of MV from BG-R46 and channels 4–6 from DSM 17938. The samples were immobilized for 45 min, and excess sample fluid was removed from the wells. The channels were then washed with 100 μl PBS, using a manual pipette. Surface shaving (limited proteolysis) of the MV fractions was performed using a trypsin for digestion of the exposed surface proteins. One hundred μl of trypsin solution (20 μg/ml in PBS) was injected into each channel and excess fluid was removed from the wells. Samples were digested for 15 min at RT and the peptides were subsequently collected by eluting 200 μl from each LPI channel. Samples were acidified with 40 μl of 10% formic acid and stored at −20°C.

Proteomic analysis

Samples were desalted (Pierce peptide desalting spin columns, Thermo Fisher Scientific) according to the manufacturer's instructions prior to analysis on a QExactive HF mass spectrometer interfaced with Easy-nLC1200 liquid chromatography system (Thermo Fisher Scientific). Peptides were trapped on an Acclaim Pepmap 100 C18 trap column (100 μm × 2 cm, particle size 5 μm, Thermo Fisher Scientific) and separated on an in-house packed analytical column (75 μm × 30 cm, particle size 3 μm, Reprosil-Pur C18, Dr. Maisch) using a gradient from 5% to 80% acetonitrile in 0.2% formic acid over 90 min at a flow of 300 nl/min. The instrument operated in data-dependent mode where the precursor ion mass spectra were acquired at a resolution of 60,000, m/z range 400–1,600. The 10 most intense ions with charge states 2 to 4 were selected for fragmentation using HCD at collision energy settings of 28. The

isolation window was set to 1.2 Da and dynamic exclusion to 20 s and 10 ppm. MS2 spectra were recorded at a resolution of 30,000 with maximum injection time set to 110 ms. The data files were searched for identification using Proteome Discoverer version 2.4 (Thermo Fisher Scientific). Since the genome of DSM 17938 is not publicly available, the genome of the parental strain *L. reuteri* ATCC 55730 (Rosander et al., 2008) was used (GenBank BioProject PRJNA30643) to identify and name the proteins. The data was matched against the ATCC 55730 genome using Mascot version 2.5.1 (Matrix Science) as a search engine. The precursor mass tolerance was set to 5 ppm and fragment mass tolerance to 50 mmu. Tryptic peptides were accepted with one missed cleavage and methionine oxidation was set as variable modification. FixedValue was used for PSM validation. The cellular localization of the detected proteins was predicted by using the information generated by Bath et al. (2005). The annotation of the proteins and identification of domains were done by using information from UniProt[1], MoonProt[2], and GenBank.[3]

Nanoparticle tracking analysis

The physicochemical characterization of MV was done by using the Nanoparticle tracking analysis (NTA). MV were diluted with PBS and directly tracked using the NanoSight NS300 system (NanoSight ™ technology, Malvern, United Kingdom). A 488 nm laser beam was used, and three videos of 90 s were recorded of each sample and triplicate histogram were averaged for each sample. Data analysis was performed using the NTA software (version 3.2).

5′-nucleotidase (5′NT) activity of MV

5′NT activity from MV was detected by using a 5′-nucleotidase assay kit (Crystal Chem High Performance Assays. USA) according to the manufacturer's instructions. The level of 5′NT activity from MV which were produced under different conditions and from different strains was quantified. All measurements were carried out as three independent experiments with biological replicates.

Detection of lipoteichoic acid (LTA) on MV

In the dot-blot assay, MV samples were loaded onto an activated and semi-dry PVDF: polyvinylidene fluoride membrane. The membrane was blocked with TBS-T:Tris-buffered saline with 0.05% Tween 20 (10 mM Tris–HCl, 150 mM NaCl pH 7.5, 0.05% Tween 20), for 1 h at room temperature after which it was incubated for 18 h at 4°C with the primary lipoteichoic acid monoclonal antibody (Thermo Fisher Scientific) at a 1:50 dilution in TBS-T with 1% BSA. The membrane was then washed with TBS-T and incubated with an HRP-conjugated secondary antibody at a dilution of 1:2,000 in TBS-T with 1% BSA, for 1 h at room temperature. After several TBS-T washes, the membrane was developed using an ECL kit (Bio-Rad Laboratories, Inc.). PBS and bacterial cells from *E. coli* were used as negative controls and LTA standards as positive control.

Immunodetection of LTA on MV by confocal microscope Zeiss LSM 780 was performed by staining MV from *L. reuteri* with PKH26 using PKH26 Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling (Sigma-Aldrich) according to the protocol. MV were loaded onto poly-L-lysine coated N.1.5 coverslips and air-dry, followed by fixation with 4% paraformaldehyde in PBS for 10 min at room temperature. Following incubation for 30 min in blocking solution (5% normal goat serum with 0.3% BSA in PBS), the samples were incubated overnight at 4°C with primary LTA monoclonal antibody (Thermo Fisher Scientific) at a 1:50 dilution in blocking solution. The coverslips were washed with PBS and incubated for 1 h at room temperature with a secondary antibody (Abberior STAR 635) at a dilution of 1:100 in blocking solution. After several PBS washes, the coverslips were mounted onto glass slides using Mowiol 4–88 and photographed by confocal microscope Zeiss LSM 780.

In vitro epithelial permeability

The human colon carcinoma cell lines (Caco-2 ATCC HTB-37) and the goblet human colorectal carcinoma cells (HT29-MTX from ECACC) were separately grown in tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% penicillin and streptomycin, at 37°C under an atmosphere of 5% CO_2 with 90% relative humidity. Caco-2 and HT29-MTX cells were grown in 25 cm^2 tissue culture flasks and split at 80%–90% confluence using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) solution. The cells were seeded at a density of 6×10^4 cells per 25 cm^2 flask.

Caco-2 and HT29-MTX cells were seeded on the apical chamber of transwell inserts (Transwell-COL; collagen-coated membrane filters) with 9:1 proportion and grown in 12-well transwell plates (Corning Costar) with a final density of 1×10^5 cells/cm^2 in each insert. Cells were maintained under the same conditions and allowed to grow for 21 days with medium (0.5 ml on the apical side and 1.5 ml on the basolateral side) that was refreshed every other day to allow the cells to become differentiated. The integrity of the cell layer was determined using two methods: transepithelial electrical resistance (TEER) and determination of fluorescein isothiocyanate-dextran (FITC-dextran) permeability. TEER was measured using the Millicell electrical resistance system (Millipore, Darmstadt,

1 https://www.uniprot.org
2 http://www.moonlightingproteins.org
3 https://www.ncbi.nlm.nih.gov/protein/

Germany). Each TEER value is the average of 6–9 independent measurements. Wells with TEER values above 250 Ω cm^2 were used for the permeability studies. Seeded Caco-2/HT29-MTX cells were pre-treated with live *L. reuteri* DSM 17938 cells (cultivated for 24 h) at 100 multiplicity of bacteria (MOB) or MV from *L. reuteri* at 10–200 multiplicity of MV (MOM) for 6 h before challenge with ETEC (enterotoxigenic *E. coli* strain 853/67, known for having a disruptive effect on epithelial integrity; Holmgren, 1973) at 100 multiplicity of infection (MOI) for an additional 6 h. TEER was measured before pre-treatment and challenge with ETEC, followed by measurement every second hour during the entire challenge. To quantify the paracellular permeability of monolayers, 1 mg/ml of 4 kDa FITC-dextran (Sigma) was added to the apical side of the inserts at the start of the challenge with ETEC. Samples from the basolateral compartment were taken after 6 h of incubation. The diffused fluorescent tracer was then analyzed by fluorometry (excitation, 485 nm; emission, 520 nm) using a FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany).

Staining of MV and localization of MV in Caco-2/HT29-MTX cell co-cultures

The MV and control were stained with PKH26 Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling (Sigma-Aldrich). For the control sample, particle-free PBS was used as the input instead of the MV standard. MV and control samples were pelleted by ultracentrifugation (Optima X Series, Beckman coulter, IN, United States) at 190,000 × *g* for 2 h at 4°C. The pellet was gently resuspended in 100 µl PBS and MV were diluted to 6 ml with PBS and placed onto 1.5 ml 0.971 M sucrose cushion. The MV were pelleted by ultracentrifugation at 190,000 × *g* for 2 h at 4°C. The pellets were gently washed with PBS and resuspended in PBS, followed by transfer to an Amicon 10 kDa MWCO filter column that was repeatedly centrifuged at 3,000 x *g* for 40 min at 4°C to reduce the volume to 0.5–1 ml. Caco-2/HT29-MTX co-cultured cells were grown on transwell filters in 12-well tissue culture plates as described above. On day 21, upon which the monolayer reached polarization, cells were either apically treated with stained MV for 6 h or left untreated. Filters were fixed in 4% paraformaldehyde for 15 min at 4°C and permeabilized with 0.2% Triton-X-100 for 15 min at RT. Membranes were rinsed with PBS. The membrane with cells was then incubated in a 3% BSA solution (Sigma Aldrich) for 1 h at RT. Mouse monoclonal anti-ZO-1 (diluted at 1:100; N-term; Invitrogen, Carlsbad, CA) was applied as primary antibody and Alexa Fluor 488 goat anti-mouse (green) as the secondary antibody (diluted at 1:200; Invitrogen). The nuclei were stained with DAPI (Invitrogen). Images were acquired using laser scanning confocal microscopy (Zeiss LSM 780 with a 63× objective; Zeiss ZEN software; Zeiss, Oberkochen, Germany).

PBMC stimulations

Healthy, anonymous, adult volunteers were included in this study, which was approved by the Regional Ethics Committee at the Karolinska Institute, Stockholm, Sweden [Dnr 2014/2052–32]. All methods were carried out in accordance with approved guidelines and all study subjects gave their informed written consent. Venous blood was collected and diluted 1:1 with RPMI-1640 cell culture medium supplemented with 20 mM HEPES (HyClone Laboratories, Inc.). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (GE Healthcare Bio-Sciences AB) gradient separation. The isolated PBMC were washed and resuspended in freezing medium containing 40% RPMI-1640, 50% fetal bovine serum (FBS; Sigma Aldrich) and 10% DMSO (Sigma-Aldrich), frozen gradually at-80°C in freezing containers (Mr. Frosty, Nalgene Cryo 1°C; Nalge CO.) and finally stored in liquid nitrogen until used in assays.

PBMC were thawed, washed, and stained with Trypan blue followed by live cell counting using a 40x light microscope. Cells were resuspended in cell culture medium containing RPMI-1640 supplemented with HEPES (20 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM; all from HyClone Laboratories, Inc.) and FBS 10% (Sigma Aldrich) at a final concentration of 1×10^6 cells/ml. Cells were seeded in flat bottomed 96-well cell culture plates at 2.5×10^5 cells/well and incubated for 48 h at 37°C with 5% CO_2 atmosphere. *Staphylococcus aureus* (*S. aureus*) 161:2 (Haileselassie et al., 2013) -cell free supernatant (CFS) was used as a stimulus at 2.5% (v/v) and lactobacilli-MV were used at a MV-to-cell ratio of 500:1. Finally, the cell culture supernatants were collected by centrifugation and stored at-20°C.

Enzyme-linked immunosorbent assay

Secreted levels of the cytokines IL-1β, IL-6, IFN-γ and TNF-α in cell culture supernatants were determined using sandwich ELISA kits (MabTech AB) according to the manufacturer's instructions. Absorbance was measured at a wavelength of 405 nm using a microplate reader (Molecular Devices Corp.) and results analyzed using SoftMax Pro 5.2 rev C (Molecular Devices Corp.).

TRPV1 antagonistic potential in primary rDRGs

The experimental procedures were conducted under ethical permit no. 76-2013 and in accordance with European and Swedish animal welfare regulations. Cultures of primary rat dorsal root ganglia cells derived from 6-week-old male Sprague–Dawley rats were obtained by microsurgical dissection, performed at the University of Gothenburg, after which the rDRGs were grown in 384-well plates. The cells were stained with the Ca^{2+} indicator Ca5 (FLIPR Calcium 5 Assay Kit, Molecular devices, CA, USA) on the

experiment day to measure intracellular calcium ion flux. MV suspended in culture medium containing Neurobasal A (Gibco, Camarillo, CA, USA) supplemented with supplement B27 (Invitrogen, Grand Island, NE, USA) and Glutamax (Thermo Fisher Scientific, Agawam, MA, USA) were added in 6 concentrations (diluted in steps 1:3) 1 h before adding the agonist capsaicin. The highest concentration of vesicles was 10% of the stock which corresponded to approximately 10^9 particles/ml. Measurements were taken in the Cellaxess Elektra discovery platform where the calcium probe intensity was measured continuously. Experiments were conducted in three replicates at two or three separate time points, $n = 9$ for DSM 17938 24 h, DSM 17938 48 h, BG-R46 48 h, LGG 24 h, and $n = 6$ for BG-R46 24 h. AMG517, a known TRPV1 antagonist, were used as control substance to verify the antagonistic effect.

Statistical analysis

Data are generally expressed as means and standard deviations, except for the immunological data which is displayed as median with interquartile range. The difference among groups was analyzed by one-way analysis of variance (ANOVA) unless stated otherwise. The significant difference was set at $p < 0.05$. In the epithelial integrity experiment comparing bacterial cells and MV, Welch's ANOVA was used. A two-way ANOVA was used for the FITC-measurements in the epithelial integrity experiment comparing different concentrations of MV. For naïve PBMC cytokine secretion, Mann–Whitney statistical test was used. Wilcoxon matched pairs signed rank test was performed for the relative concentrations of TNF-α and IFN-γ. A mixed effects model was used in the TRPV1-model.

Results

Production parameters and strain features affect MV characteristics

To increase the general knowledge of MV from *L. reuteri* DSM 17938, we initially cultivated DSM 17938 under standard conditions for 24 h. Cultivation time affected the survival and appearance of the bacterial cells, and after 24 h of cultivation most bacteria were alive, but the viability had dropped approximately 30 times after 48 h (Supplementary Figure S1). The bacterial cells were imaged by SEM showing a large number of budding vesicles on the cell surface (Figure 1A). As the next step we studied the production of MV during different culture conditions and performed a physiochemical characterization of the MV. The production was affected by the culture conditions and both prolonged cultivation time (48 h) and oxygen stress resulted in a 16-fold increase of MV (Table 1). We also investigated the strain *L. reuteri* BG-R46, which was found to produce approximately the same amount of MV as DSM 17938. We discovered that the MV contained protein, RNA and DNA. The protein concentrations of *L. reuteri* DSM 17938 derived MV from 48 h-cultures and oxygen stressed bacteria were 2-fold higher than those from the 24 h-cultures ($p < 0.05$; Table 1). SEM analyses revealed that the bacterial cells from both strains were intact after 24 h and multiple vesicles per cell were observed (Figures 1B,C). However, both the extended cultivation time (48 h) and the oxygen stress led to disintegration of bacterial cells (Figures 2D,E). MV appeared on the bacterial surface and were also released from the bacterial cells (Figures 1A–E). Analysis of the MV by TEM showed that they had a broad particle distribution, polymorphic structure, and spherical shape (Figures 1F,G). The size-heterogeneity of the vesicles was confirmed with nanoparticle tracking analysis, which revealed a wide size distribution (MV's diameter from 20 nm up to 500 nm) of the isolated MV (Figure 2). The size profiles of MV from the two strains were quite similar after 24 h, but there were broader size distributions of MV from 48 h and after O_2 stress. The Nanoparticle Tracking Analysis (NTA) analysis detected the presence of larger particles which may be aggregates of MV (Figure 2).

Proteome analysis and surface characteristics of MV

To further investigate the protein exposed on the MV, we analyzed MV isolated from both DSM 17938 and BG-R46 using liquid chromatography tandem mass spectrometry (LC–MS/MS). More than 800 proteins were identified (data not shown), and the vast majority of the proteins with highest #PSM and #peptides scores were predicted to be secreted (Supplementary Table S1). It means that they normally would be predicted to be localized on the bacterial cell surface or being released from the bacteria, and many of those proteins are tentatively involved in host-bacterial interactions (Supplementary Table S1). Several of the proteins are predicted to be involved in adhesion to cells and mucus; a LPXTG domain protein with a Rib/alpha-like repeat (HMPREF0538_20063), a MucBP protein (HMPREF0538_20356), an YSIRK signal peptide protein with 9 Rib/alpha-like repeats (HMPREF0538_20775–20,774), and the earlier described collagen/mucus binding protein CnBP (HMPREF0538_21501). In addition, several moonlighting proteins were detected, most of which were predicted to be involved in adhesion. The MV preparations also contained proteins predicted to be involved in production of extracellular polysaccharides (EPS). One of the most abundant proteins was a dextran sucrase (HMPREF0538_20764) and several proteins expressed from a big EPS operon were detected (HMPREF0538_20363, HMPREF0538_20382, HMPREF0538_20383). Another abundant protein detected in the MV preparations was a LPxTG anchored 5′-nucleotidase (5′NT; HMPREF0538_20056). This enzyme converts adenosine monophosphate (AMP) to adenosine and this activity was confirmed with an enzymatic assay (Table 2). The 5′NT activities

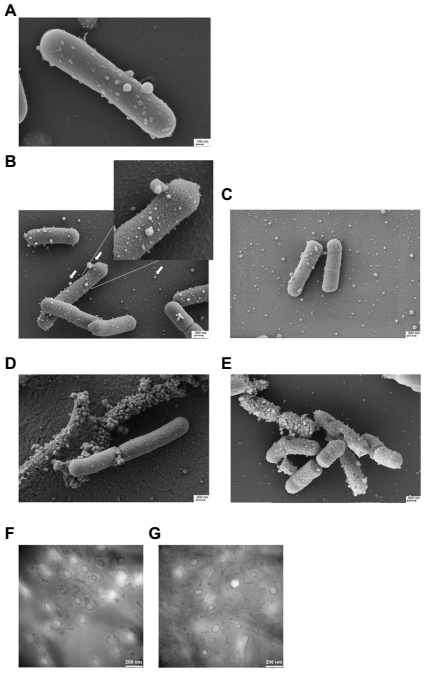

FIGURE 1
Scanning electron microscopy images of *L. reuteri* cultivated under different conditions. *L. reuteri* DSM 17938 cultivated in MRS for 24 h with extracellular membrane vesicles of varying sizes budding of the bacterial surface **(A)**. *L. reuteri* DSM 17938 after 24 h with **(D)**/without O_2 stress condition **(B)** and 48 h **(E)** of cultivation; BG-R46 after 24 h cultivation **(C)**. A representative example of three independent experiments is shown. Magnification 40k. Arrows indicate MV budding from the cell surface and released MV. Transmission electron microscopy negative staining analysis of *L. reuteri* DSM 17938 extracellular membrane vesicles isolated after 24 h **(F)** and 48 h **(G)** of cultivation.

of MV from 48 h (DSM 17938) and oxygen stressed condition were more than 15-fold higher compared to the activity at 24 h (DSM 17938) which is in line with the MV concentration ratios described above. Interestingly, BG-R46 derived MV had more than 7-fold higher 5′NT activity compared to the corresponding DSM 17938 preparation, although they had the same concentration of MV. 5′NT activity from *L. rhamnosus* GG (LGG) derived MV was not detectable. Proteins involved in cell wall modulation was another abundant group. Both proteins involved in synthesis of peptidoglycan (penicillin-binding proteins; Pbp2b and Pbp1a) and degrading peptidoglycan (HMPREF0538_20363, HMPREF0538_21064) were detected. Also, a protein involved in LTA biosynthesis (HMPREF0538_21428) was found. Even though the proteomic approach used here was for general protein identification, i.e., not quantitative proteomics, some proteins seem to be present to a greater extent (#peptides and #PSMs) in DSM 17938 vesicles than in BG-R46 vesicles. Interestingly, one of those proteins were the above-mentioned 5′NT. The top 100 most abundant proteins sorted by #PSM showed a 72% overlap between DSM 17938 and BG-R46 bacterial surfaces. Similarly, the overlap between the two strains MV were 62%. Meanwhile, among the top 100 proteins sorted by #PSM, there was only 21% identity between DSM 17938 bacterial surface and DSM 17938 membrane vesicle surface and 25% between BG-R46 bacterial surface and BG-R46 membrane vesicle surface (Figure 3). This indicates that there is a large difference between what is present on the bacterial and MV surfaces.

We also investigated if LTA was present on MV. The dot blot assay showed that MV derived from both *L. reuteri* DSM 17938 and BG-R46 carried LTA (Figure 4A). Also, according to confocal immunofluorescence microscope images, LTA was detected on MV (Figure 4B). All the evidence indicated that *L. reuteri* DSM 17938 and BG-R46 derived MV have LTA exposed on the surface. LTA was used as positive control (Figure 4A) and *E. coli* cells as a negative control (data not shown). A schematic image of MV with its tentatively bioactive molecules is presented in Supplementary Figure S2.

Limosilactobacillus reuteri and its derived MV protect epithelial barrier integrity from the detrimental effect of enterotoxigenic *Escherichia coli* (ETEC)

Next, we wanted to investigate whether *L. reuteri* derived MV could protect epithelial barrier integrity of monolayers of cultivated epithelial cells. We used a model with a mix of Caco-2 and HT29-MTX cells. The cells were exposed to enterotoxigenic *E. coli* (ETEC) which induced a strong reduction in transepithelial electrical resistance (TEER), but pre-treatment with either *L. reuteri* bacterial cells or MV from *L. reuteri* provided a protective effect against this reduction (Figure 5A). Furthermore, a FITC-dextran flux experiment demonstrated that both MV and bacterial cells decreased the ETEC induced leakage of the macromolecule. When we pre-treated the Caco-2/HT29-MTX co-cultures with MV (multiplicity of MV per epithelial cell of 200:1), the leakage caused by ETEC decreased approximately by 65% (Figure 5B).

We also compared the effects of the different MV preparations, and already at MOM 10 all variants except DSM 17938 24 h gave a protective effect (Figure 5B). At MOM 50–200 all variants protected. MV from *L. reuteri* BG-R46 gave a significantly better protection than DSM 17938 24 h at all doses except MOM 200 (Figure 5B). However, all MV gave the same protection level at MOM 200. The MV preparations protected the epithelial cells against ETEC challenge in a dose-dependent manner.

We further investigated the interaction between MV and Caco-2/HT29-MTX cells by using confocal microscopy.

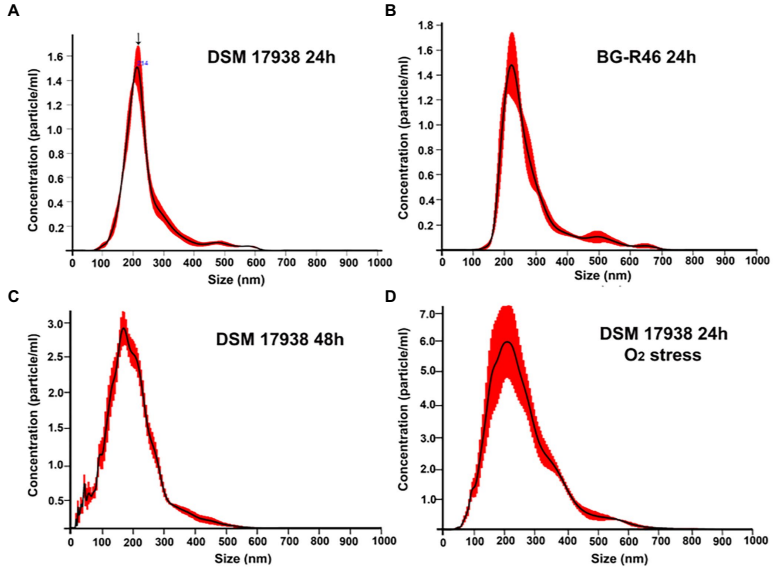

FIGURE 2
Physicochemical characterization of *L. reuteri* derived MV through NanoSight tracking analysis (NTA). The nanoparticle tracking distribution of *L. reuteri* DSM 17938 derived MV after 24 h, 48 h, under O_2 stress of cultivation and isolated from BG-R46 were shown in **(A,C,D,B)** individually. Figures are representative of three independent triplicates. The size distribution represents the wide distribution of MV for each MV prep. Arrow shows the mean peaks of particles at 214 nm **(A)**. Figure 2A was originally published in Scientific Reports (Mata Forsberg et al., 2019).

TABLE 1 Quantification of *L. reuteri* derived extracellular membrane vesicles (MV) and their content of DNA, RNA, and protein.

MV content	DSM 17938 24 h	BG-R46 24 h	DSM 17938 O_2 stress 24 h	DSM 17938 48 h
MV conc. (particles/ml)	$3.0 \pm 1.9 \times 10^{9a}$	$3.0 \pm 2.7 \times 10^{9a}$	$5.3 \pm 1.1 \times 10^{10b}$	$4.9 \pm 2.6 \times 10^{10b}$
dsDNA (μg/ml)	0.51 ± 0.3^{a}	0.54 ± 0.1^{a}	1.26 ± 0.4^{a}	0.68 ± 0.01^{a}
RNA (μg/ml)	2.27 ± 0.8^{a}	2.65 ± 0.25^{a}	3.46 ± 0.2^{a}	3.28 ± 0.4^{a}
Protein (μg/ml)	183.5 ± 30^{a}	216.25 ± 28^{a}	506 ± 20^{b}	462.5 ± 50^{b}

Value with the same letter is not significantly different from one another determined by one-way analysis of variance (ANOVA), $p < 0.05$. All values are mean ($n = 3$), error bars are SD ($n = 3$). [a,b]indicate statistical differences between samples.

Interestingly, MV were taken up and could be detected inside the epithelial cells or potentially having a paracellular location (Figure 6; Supplementary Video S1).

MV from *Limosilactobacillus reuteri* strains modulate cytokine production from human PBMC cultures

Subsequently, we investigated how the MV preparations affected the stimulatory potential of peripheral blood mononuclear cells (PBMC). The immune cells were cultured for 48 h in the presence of MV isolated from lactobacilli grown for 24 or 48 h. Interestingly, DSM 17938, BG-R46 and LGG derived MV harvested after 24 h induced interleukin (IL)-6 and IL-1β. MV isolated from DSM 17938 and BG-R46 grown for 48 h induced to a lower extent secretion of IL-6, while IL-1β was not affected by cultivation time (Figures 7A,B). We further focused on MV derived after 48 h to investigate the ability of MV to regulate cytokine responses induced by a potential pathogen. MV were added to *S. aureus* cell free supernatant (CFS) challenged PBMC cultures followed by cytokine measurement. Addition of *L. reuteri*-derived MV to *S. aureus*-stimulated PBMC significantly blocked secretion of the proinflammatory cytokine IFN-γ (Figure 7C) and reduced the secretion of TNF-α (Figure 7D), whereas the MV derived from LGG did not decrease the *S. aureus*-induced secretion of IFN-γ and TNF-α.

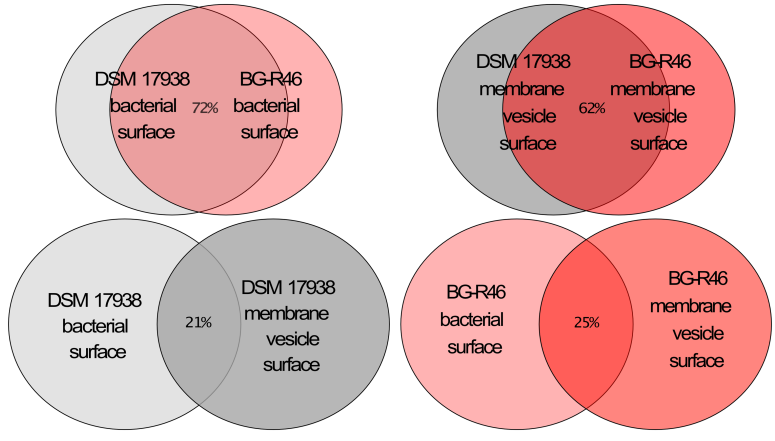

FIGURE 3
Venn diagram displaying a comparison of the 100 most abundant proteins on the bacterial surfaces and the membrane vesicle surfaces of DSM 17938 and BG-R46. The proteomic analysis was performed by using a trypsin mediated surface shaving, Lipid-based Protein Immobilization method. The color key is gray=DSM 17938 and red=BG-R46, light color indicate bacterial surface, dark color indicate membrane vesicle surface.

MV from *Limosilactobacillus reuteri* antagonize the TRPV1 receptor

TRPV1 has been suggested to be involved in pain perception in infantile colic. Therefore, we studied the effect of MV from *L. reuteri* in a rat dorsal root ganglion cells TRPV1 model. The antagonistic effect in % was calculated from the fluorescence intensity of the Ca^{2+} indicator Ca5. A dose dependency in TRPV1 antagonism was observed in response to MV preparations from both DSM 17938 and BG-R46, diluted 10, 30 and 90 times. Control vesicles derived from LGG did not exhibit this antagonistic effect. These results showed that MV from both DSM 17938 and BG-R46 exhibit a significantly stronger antagonistic effect on the TRPV1 receptor compared to the LGG vesicles (Figure 8).

Discussion

We isolated MV from *L. reuteri* cultivated under different conditions and this resulted in differences in yield and different morphologies (Figure 1). The yield increased with longer cultivation time and in response to oxygen stress and this correlated with fewer live bacteria (Supplementary Figure S1). In concordance with that, more vesicles appeared on the surface of partly degraded cells (Figures 1D,E). Membrane fragments from dead cells can vesicularize and consequently increase the amount of MV, a phenomenon that previously has been described (Toyofuku et al., 2017). However, the SEM analysis (Figure 1) showed vesicles budding on the surface of intact *L. reuteri* cells, indicating that there is a MV biogenesis mechanism that is independent of cell death, a process that also have been reported by others (Brown et al., 2015; Orench-Rivera and Kuehn, 2016). Altogether, this indicates the existence of subpopulations of MV, and future studies could reveal if those have different content and functions.

Although the studies regarding MV production in Gram-positive bacteria has intensified, the mechanisms of vesiculogenesis and transport through the cell wall remains poorly understood. It has been discussed if cell wall modifying enzymes play a role in the MV release through the Gram-positive cell wall (Brown et al., 2015; Toyofuku et al., 2017) and the results from the proteome analysis support this hypothesis. First, MV from *L. reuteri* DSM 17938 carry several peptidoglycan-degrading enzymes (Supplementary Table S1), which potentially could generate channels through the cell wall. Through these channels, cytoplasmic membrane material might be forced by turgor pressure to protrude into the extracellular space and thereafter released as MV. Furthermore, the MV also carry several transpeptidases (Supplementary Table S1) that possibly could be involved in healing of the cell wall after the vesicular release. Thus, the controlled release of MV from *L. reuteri* DSM 17938 could potentially be facilitated by both peptidoglycan degrading and biosynthesis enzymes.

Previous studies have shown that MV can carry a wide range of cargo, including DNA, RNA and proteins (Habier et al., 2018), potentially having the ability to deliver combinatorial information to different types of cells in their microenvironment (Skog et al.,

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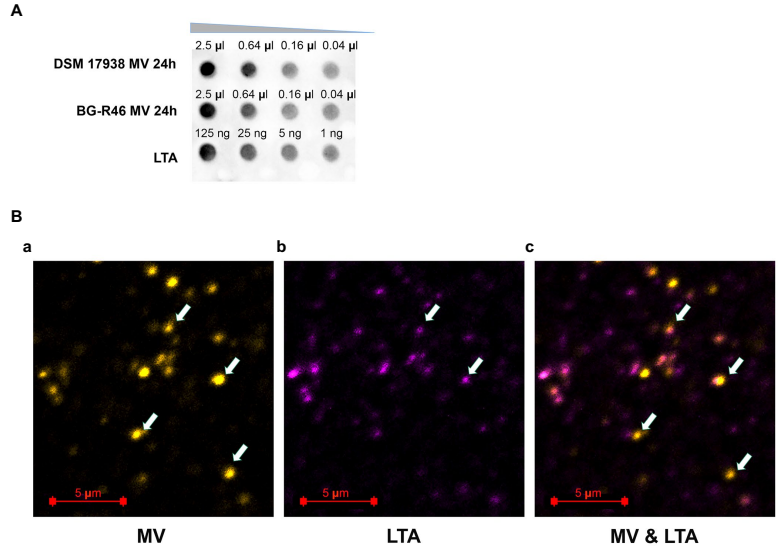

FIGURE 4
Lipoteichoic acid (LTA) presents on *L. reuteri* DSM 17938 and BG-R46 derived MV. **(A)** Dot-blot assay using the primary antibody against lipoteichoic acid (Thermo Fisher Scientific). The samples were loaded onto an activated and semi-dry PVDF membrane with different amount. The concentrations for MV from *L. reuteri* DSM 17938 and BG-R46 were 4.4×10^9 particles/ml. **(B)** Confocal immunofluorescence microscopy images of MV from *L. reuteri* DSM 17938. MV were stained by PKH26 (yellow color) **(a)** and LTA were immunolabeled by a primary antibody against lipoteichoic acid (Thermofisher) and a secondary antibody (Abberior STAR 635) which is seen as violet color **(b)**; **(c)** is the superimposing of these two labels. The arrow indicates the MV in the images. MV aggregates were also observed in these images (big dots).

TABLE 2 5′-nucleotidase activity of MV which was isolated from *L. reuteri* under different cultivation conditions.

MV	24 h DSM 17938	24 h BG-R46	48 h DSM 17938	24 h DSM 17938 O_2 stress	24 h LGG
5′-nucleotidase activity (U/1 × 10^{12} MV)	3.8 ± 0.6^a	27.3 ± 4.1^b	3.2 ± 0.6^a	3.5 ± 0.4^a	Not detectable
5′-nucleotidase activity (U/L)	11.4 ± 1.2	82 ± 11	169.9 ± 6.8	170.3 ± 9.6	Not detectable

24 h DSM 17938, 48 h DSM 17938 denote MV from *L. reuteri* DSM 17938 harvested at 24 and 48 h; 24 h DSM 17938 O_2 stress denotes MV from *L. reuteri* DSM 17938 cultivated under O_2 stress and harvested at 24 h; 24 h BG-R46 denotes MV from *L. reuteri* BG-R46 and harvested at 24 h. Values with different letters are significantly different determined by one-way analysis of variance (ANOVA), $p < 0.05$. Data are given as means three independent measurements. Error bars are SD ($n = 3$).

2008). It has been shown that MV from pathogenic *S. aureus* carry RNA and DNA, which are protected from degradation, and that these molecules play an important role in virulence and immune modulation through their transmission of signals to host cells (Lee, 2019; Rodriguez and Kuehn, 2020). MV from *L. reuteri* contain both RNA and DNA and detailed investigations of the nucleic acids content of *L. reuteri* derived MV will be a subject for future studies. As already mentioned, MV preparations from *L. reuteri* also contained proteins (Table 1) and after performing a proteome analysis it was concluded that they represent a wide array of functions (Supplementary Table S1). Interestingly, a majority of the most abundant proteins were predicted to be localized on the cell surface, which differ from other studies of MV from lactobacilli and other potentially probiotic bacteria (Al-Nedawi et al., 2015; De Rezende Rodovalho et al., 2020; Hu et al., 2021). Besides the cell wall biogenesis proteins discussed above, several of the surface proteins are predicted to be involved in host–microbe interactions. A key feature for many bacteria is adhesion to surfaces and this is believed to be true also for a probiotic bacterium (Muscariello et al., 2020). Binding to the mucosal surface will both prolong the persistence in the intestine and enable a closer contact with enterocytes and immune cells with which the bacterium could interact. Some proteins to highlight are (i) the collagen/mucus binding protein CnBP that has been shown to be an important adhesion factor for *L. reuteri* (Roos et al., 1996; Velez et al., 2007); (ii) the large Rib motif containing protein Lr1694, a type of protein that has been described to induce protective immunity and promote binding to human epithelial cells of streptococci (Stalhammar-Carlemalm et al., 1999); and (iii) Lr1612 a large MucBP protein, containing

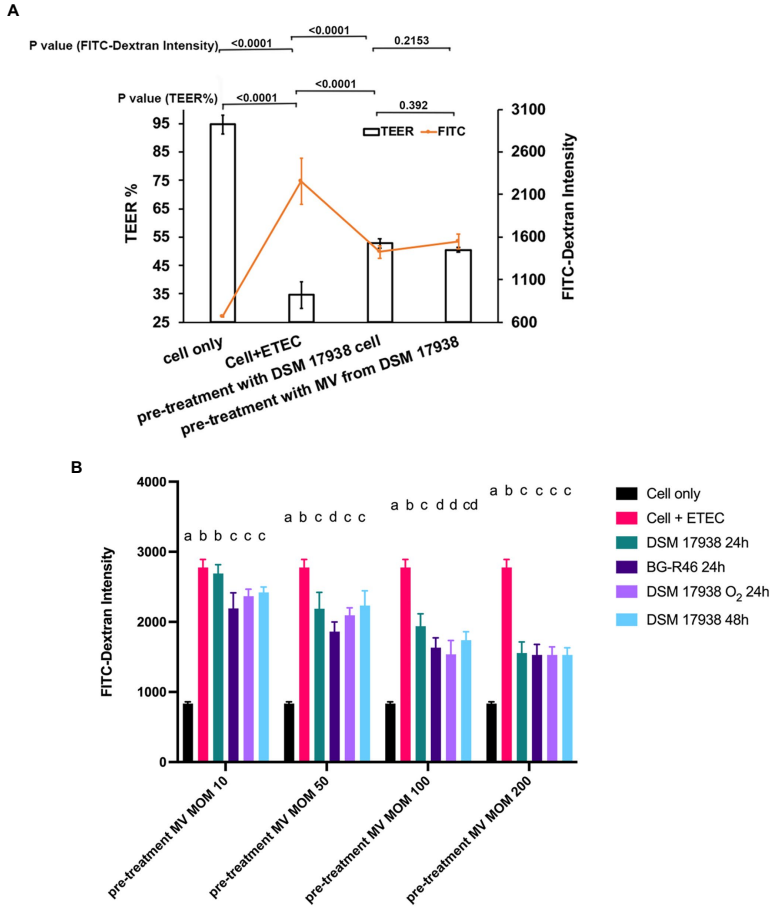

FIGURE 5
Effect of *L. reuteri* derived MV on the barrier integrity of monolayers treated with ETEC. The Caco-2/HT29-MTX co-cultures were grown on transwell filters for 21 days. **(A)** Polarized monolayers with/without pre-treatment of *L. reuteri* DSM 17938 bacteria cells and derived MV at multiplicity of bacteria/MV of 100:1 and 200:1 for 6 h. After 6 h pre-treatment, the monolayer was infected with ETEC at multiplicity of infection (MOI) 100 for 6 h. TEER was measured in ohms and corrected for the resistance of the blank filters and for the membrane area and expressed as percentage of the starting value (before pre-treatment with MV). The fluorescence intensity is from the fluorescein isothiocyanate (FITC)-dextran which was detected from the basolateral pole. Data are given as means of six independent seedings. Statistical analysis by one-way analysis of variance (ANOVA), $p<0.05$. **(B)** Polarized monolayers with/without pre-treatment of MV which were isolated from *L. reuteri* under different cultivation conditions. The fluorescence intensity is from the FITC-dextran which was detected from basolateral pole of each sample. Columns with different letters are significantly different ($p<0.05$).. Statistical analysis by two-way ANOVA. Error bars are SD ($n=9$).

motifs that have shown to be involved in adhesion to mucus and cells (Roos and Jonsson, 2002; Van Tassell and Miller, 2011). It is intriguing that also the MV from *L. reuteri* carry those adhesion proteins, and it both supports the results from the cell models (e.g., Figure 6) and open for further studies of intimate interactions between MV and host cells. Another set of interesting proteins present on the surface of the membrane vesicles are the relatively high number of moonlighting proteins which has been described in other bacteria, i.e., proteins with dual functions intracellularly and extracellularly (Kainulainen and Korhonen, 2014). The vesicles possess a number of these and the fact that they are found with a surface shaving-based proteomics method emphasizes their moonlighting function. Among the moonlighting proteins we found enolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, glucose 6-phosphate isomerase, elongation factor G and endopeptidase O. These proteins have been shown to

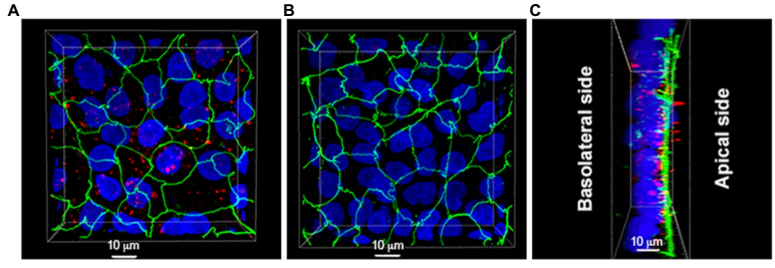

FIGURE 6
Localization of MV derived from *L. reuteri* DSM 17938 in Caco-2/HT29-MTX cells. **(A)** Caco-2/HT29-MTX cells were pretreated with MV for 6 h. **(B)** Caco-2/HT29-MTX cells were pretreated with PBS buffer which was used for resuspension of MV for 6 h. **(C)** Most of MV were taken up by Caco-2/HT29-MTX cells. Unbound vesicles were washed away prior to the experiment. Images are representatives of four separate experiments, color key: blue=nuclei, green=ZO-1, red=MV.

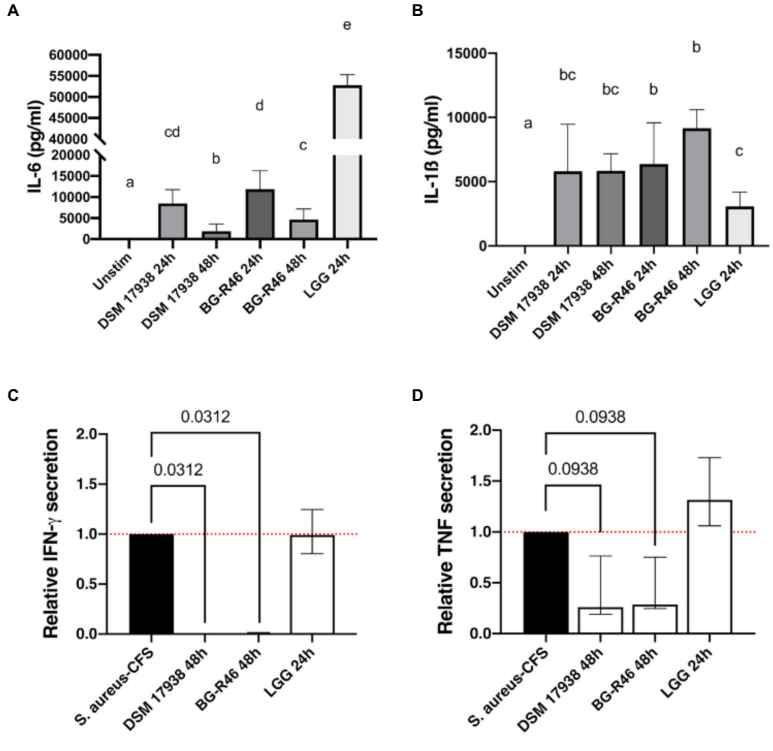

FIGURE 7
Immunomodulatory effects of *L. reuteri* DSM 17938, BG-R46 and LGG derived MV in PBMC. Secretion of cytokines IL-6 **(A)** and IL-1β **(B)** in naïve PBMC after incubation with MV derived from DSM 17938, BG-R46 and LGG. PBMC were cultured with MV for 48h before quantification. Bar plots show median with interquartile range. Bars with different letters are significantly different ($p<0.05$). Statistical analysis by Mann–Whitney test, $n=6$. Relative secretion of IFN-γ **(C)** and TNF-α **(D)** in *S. aureus* stimulated-PBMC in response to incubation with MV derived from *L. reuteri* DSM 17938 and BG-R46 and LGG. PBMC were cultured with MV for 48h before quantification. Quantification of IFN-γ and TNF-α levels from PBMC stimulated with *S. aureus* were set to 1. Statistical analysis by Wilcoxon matched pairs signed rank test, $n=6$.

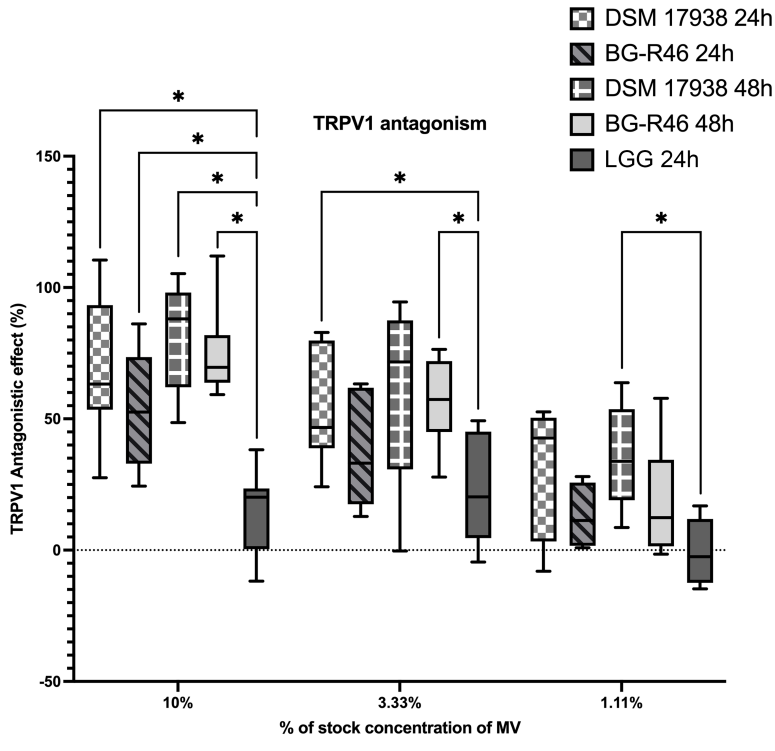

FIGURE 8
TRPV1 antagonistic effect of extracellular membrane vesicles in primary rat dorsal root ganglion cells (rDRGs). Primary rDRGs were incubated with membrane vesicles in three different concentrations (10%, 3.33%, 1.11%) for 1h before the experiment. Capsaicin was added and Ca^{2+} was measured and antagonistic response was calculated. Data show that *L. reuteri* DSM 17938 and BG-R46 derived MV antagonize TRPV1 in a dose dependent manner. Box plot with min to max and average value representation. DSM 17938 24h denote MV from *L. reuteri* DSM 17938 24h; DSM 17938 48h denote MV from *L. reuteri* DSM 17938 48h; BG-R46 24h denotes MV from *L. reuteri* BG-R46 harvested at 24h; BG-R46 48h denotes MV from *L. reuteri* BG-R46 harvested at 48h. Box plot with min to max and average value representation. N=9 for DSM 17938, DSM 17938, BG-R46 48h. N=6 for BG-R46 24h. Asterix * indicates p<0.05.

be highly involved in host interactions by facilitating mucus- and epithelial cell- binding functions extracellularly (MoonProt[4]). Similarly, moonlighting proteins have previously been shown to be in high abundance on outer membrane vesicles from Gram-negative bacteria (Dineshkumar et al., 2020).

Already in 1967 it was observed that extracellular products from pathogen bacteria could be engaged in immunomodulatory activities (Chatterjee and Das, 1967). It is also well known that pathogen derived MV can be detrimental and disturb the GI tract homeostasis (Ismail et al., 2003; Bielaszewska et al., 2013). On the contrary, MV from some microbes may help to maintain the homeostasis of the GI tract. MV from both probiotic *E. coli* Nissle 1917 and ECOR63 have been found to upregulate the expression of tight junction proteins and by that strengthen the barrier of intestinal epithelial cells (IECs; Alvarez et al., 2016). It has been reported that *L. reuteri* protect intestinal epithelial monolayers by downregulating expression of IL-6 and TNF-α, induce cytoprotective heat shock proteins (HSP), increase expression of tight junction protein and decrease the adhesion of pathogens (Liu et al., 2015; Karimi et al., 2018). In the current study, it was shown that *L. reuteri* bacterial cells and also their derived MV can protect the epithelial barrier integrity of Caco-2/HT29-MTX co-cultures, which simulates the human intestinal mucosa, against the detrimental effect of ETEC. This effect was achieved with all MV preparations, however MV from strain BG-R46 gave significantly better protection than MV from DSM 17938 (Figure 5B). Although the mechanism of protection is currently unknown the MV

4 http://www.moonlightingproteins.org

carry two types of molecules, LTA and 5′ nucleotidase (5′NT), with a potential link to epithelial protection. LTA detected on the surface of the MV (Figure 4) has in several studies been described to protect the epithelial barrier *via* interactions with TLR2 (Gu et al., 2016). 5′NT was detected both in the proteome analysis (Supplementary Table S1) and by measuring the enzyme activity (Table 2). This extracellular protein converts AMP to adenosine, which is a potent signal molecule that among other functions has a role in strengthening tissue barriers *via* interacting with the A2a and A2b receptors (Madara et al., 1993; Strohmeier et al., 1997; Lennon et al., 1998). Interestingly, at a multiplicity of MV per epithelial cell (MOM) of 10, MV from *L. reuteri* BG-R46, which has the highest 5′NT activity, showed the strongest effect on epithelial cell integrity (Figure 5B). However, this was not supported by the proteomic analysis. A possible explanation for this discrepancy could be that the 5′NT is exposed differently on the two strains and steric hindrance thereby prevent the trypsin cleavage used in the surface shaving protocol. It should be highlighted that the proteomics analysis included in this study was not performed for quantitative measures. We further investigated how MV interacts with intestinal epithelial cells, and with confocal microscopy we demonstrated that MV were taken up by the cells. Thus, MV could potentially interact with apical, intracellular or basolateral targets of epithelial cells. These interactions may also mediate transportation of vesicles *in vivo* to distant parts of the body. A recent review (Chronopoulos and Kalluri, 2020) discuss bacterial membrane vesicle presence in the blood as well as their dissemination throughout the body and tentative access to the brain. Interestingly, adenosine receptor signaling has been described as an access route through the blood brain barrier (Bynoe et al., 2015). Further studies addressing systemic MV effects is warranted.

The immunostimulatory activity of lactobacilli is both species and strain dependent. MV from both *L. reuteri* DSM 17938 and *L. reuteri* BG-R46 showed immunostimulatory activity by inducing secretion of IL-6 and IL-1β in PBMC (Figure 7), two cytokines described as markers for immune maturation in infants (Rabe et al., 2020). Interestingly, MV from both strains also blocked the secretion of IFN-γ and reduced secretion of TNF-α induced by *S. aureus*. Thus, potentially *L. reuteri* derived MV can modulate the immune system, both by stimulating basal immune responses and dampen pathogen-induced inflammation. MV isolated from LGG stimulated IL-6 and IL-1β but did not reduce IFN-γ and TNF-α, suggesting that MV from different lactobacilli interact with immune cells through different mechanisms. As discussed above, the MV from *L. reuteri* carry LTA. This molecule is one of the major surface components of Gram-positive bacteria and their vesicles known to be involved in immunomodulation (Mizuno et al., 2020; Champagne-Jorgensen et al., 2021) as well as in attachment to host cells (Beachey and Courtney, 1987; Ohshima et al., 1990). Wang et al. (Wang et al., 2000) have reported that LTA from *S. aureus* induce IL-6 production in both T cells and monocytes in a human whole blood model. An interesting link to epithelial protection is that IL-6 has been described to protect the mucosal barrier by upregulating the expression of keratin-8 and keratin-18 (Wang et al., 2007), which could be an additional mechanism for how the MV enhance the epithelial barrier integrity.

The above discussed adenosine is also an important modulator of inflammation and its anti-inflammatory effects have been well established in different models (Colgan and Eltzschig, 2012; Bowser et al., 2017; Liu et al., 2018). Extracellular adenosine reduces expression of pro-inflammatory cytokines, such as TNF-α in IECs (Alam et al., 2009). This, together with the fact that MV from LGG, lacking 5′NT activity, did not dampen TNF-α, suggests that the 5'NT contributes to the observed anti-inflammatory effect. This is supported by previously observed contrasting effects of *L. reuteri* DSM 17938 and LGG in a T-reg deficient mice model where only *L. reuteri* increased the abundance of adenosine metabolites, such as inosine, in plasma (Liu et al., 2021). This altogether indicates that 5′NT located on the surface of MV, similar to CD73 on T-reg cells (Alam et al., 2009), play an important role in the regulation of mucosal immune responses. Intriguingly, CD8 T-cells have been shown to secrete CD73 positive vesicles that contribute to battling inflammation in inflamed tissues (Schneider et al., 2021).

We also detected a dextran sucrase, having the highest #PSM and #peptide values, on the surface of MV. This enzyme catalyze glycosidic bindings in sucrose and utilize the glucose molecules to synthesize extracellular polysaccharides (EPS) (Monchois et al., 1999). It has previously been described that *L. reuteri* DSM 17938 express the same dextran sucrase that was found on the MV, and that the produced EPS can inhibit ETEC from adhering to cultivated epithelial cells and reduce the proinflammatory effects of the pathogen (Ksonzekova et al., 2016). It is intriguing that also the MV have the capacity to produce EPS, and in that way add a component to the arsenal of bioactivities.

Limosilactobacillus reuteri DSM 17938 has proven to be effective for treatment of infantile colic (Urbanska and Szajewska, 2014). The etiology of colic is not fully understood, but it is plausible that visceral pain is one of the main reasons for the extensive crying in colicky infants (Geertsma and Hyams, 1989). In mouse models, DSM 17938 has previously been shown to antagonize one of the main pain receptors, namely TRPV1 (Perez-Burgos et al., 2015), and by that decrease firing of pain signals from the intestine. Here, we demonstrate that also the MV from DSM 17938 and BG-R46 are able to antagonize TRPV1, perhaps to an even greater extent than *L. reuteri* DSM 17938 cells, which was reported by Perez-Burgos et al. (2015). The authors reported a decreased TRPV1 ionic current in a similar dorsal root ganglion (DRG) model, evoked by 10^9 CFU/ml DSM 17938, with a reduction value of 42%. Here we report a reduction value of 75% evoked by the same

concentration of MV from the same strain, indicating a higher antagonistic effect of MV than from bacterial cells. The higher antagonistic effect from MV could relate to their advantageous size that could help them access the target more easily or being internalized as shown in Figure 6. Adenosine which is a result from the above-described 5′NT activity, could play an important role in TRPV1 antagonism. This effector molecule has previously been shown to antagonize the TRPV1 receptor (Puntambekar et al., 2004) but future studies are needed to clarify if this is the mechanism by which *L. reuteri* interacts with the receptor.

The etiology of infantile colic is not fully understood (Daelemans et al., 2018). However, the models used in this paper are all well connected to the hypothesis around the amelioration of infantile colic by DSM 17938 and covers mechanisms related to potential underlying disturbances (Supplementary Figure S3). Of course, those *in vitro* models are far from an infant gastrointestinal tract and whether the MV has the same functions *in vivo* remains to be elucidated. We can however conclude that *L. reuteri* MV reproduce the mechanistic actions by which strain DSM 17938 is thought to ameliorate infantile colic. Additionally, MV from strain BG-R46 have comparable and in some biological models stronger activities than MV from DSM 17938, suggesting that BG-R46 would be an interesting candidate for further evaluation in clinical trials addressing infantile colic. While MV exposed protein profiles from the two strains were highly similar, the 5′NT activity was significantly higher in BG-R46, and we hypothesize that adenosine could be an important mediator of probiotic effects. To the best of our knowledge, MV carrying the host-interaction enzymes 5′NT and glucansucrase has never been reported before. Finally, the ability of membrane vesicles (MV) from *L. reuteri* to protect epithelial cells from detrimental effects of ETEC, modulate cytokine responses and antagonize activation of TRPV1, altogether demonstrates a novel type of multifunctionality of MV from a mutualistic bacterium. These findings could contribute to the development of new innovative and more efficient probiotic or postbiotic products (Wegh et al., 2019).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material. https://osf.io/evuy2/?view_only=2946487314cb43039520855a0dd6c4e8

Ethics statement

The animal study was reviewed and approved by ethical permit no. 76–2013 and Regional Ethics Committee at the Karolinska Institute, Stockholm, Sweden Dnr 2014/2052–32, experiments were performed in accordance with European and Swedish animal welfare regulations.

Author contributions

YP and LEL: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization and writing-initial draft, review and editing. MMF and DA: data curation and formal analysis. AP: methodology. HB and ES: funding acquisition, supervision. RK: data curation, investigation, resources, visualization. HJ: conceptualization, resources, supervision. SR: conceptualization, data curation, funding acquisition, project administration, resources, supervision, validation, writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SR, LEL, and HB are all employees of BioGaia AB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1032202/full#supplementary-material

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Supplementary Material

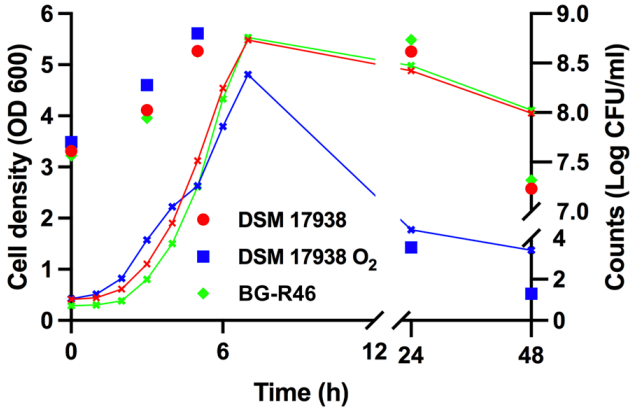

Figure 1. Growth curve of *L. reuteri* DSM 17938 cultivated in flasks with/without agitation (O_2 stress) and BG-R46 cultivated without agitation. The optical densities (crosses) were measured by using a spectrophotometer and counts of live bacteria (large symbols) were counted by plating on MRS agar plates. color key: red = DSM 17938, blue = DSM 17938 O_2, green = BG-R46.

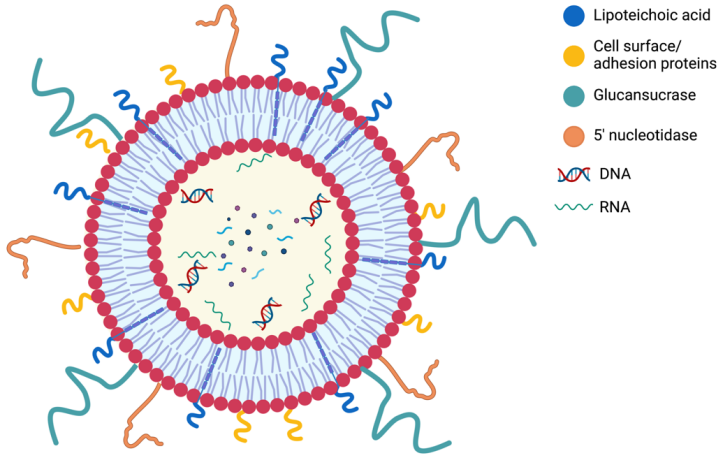

Figure 2. Schematic overview of bioactive molecules present in MV of *L. reuteri.* Color and structure code is denoted in the figure.

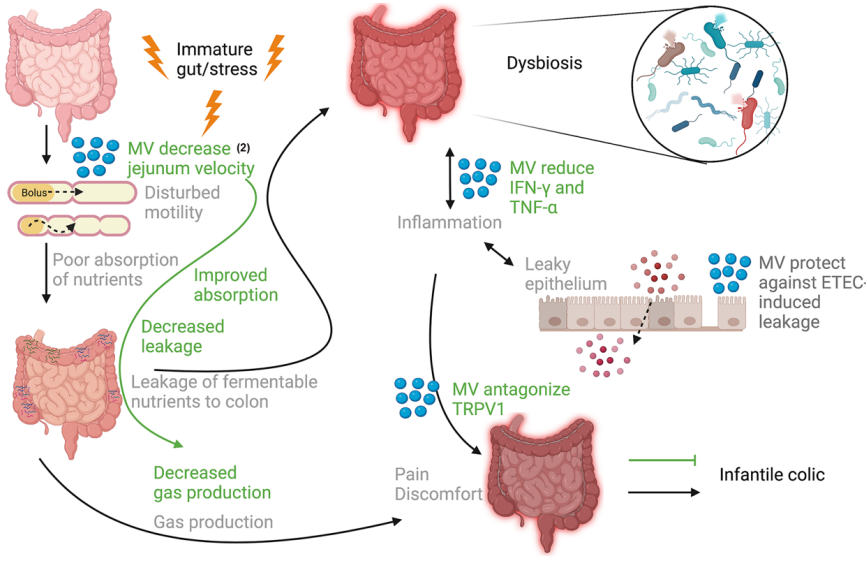

Figure 3. Schematic overview of how *L. reuteri* MV reproduce the proposed mechanism by which *L. reuteri* ameliorate infantile colic. Green text and markers indicate functions of MV while gray and black indicate the colic phenotype. (2) West, C.L., et al., Microvesicles from Lactobacillus reuteri (DSM-17938) completely reproduce modulation of gut motility by bacteria in mice. PLoS One, 2020. 15(1): p. e0225481.

Table 1. A selection of the most abundant proteins detected on MV derived from *L. reuteri* strains DSM 17938 and BG-R46 harvested at 24 h. After cultivation of the bacteria in MRS broth the MV were isolated by ultracentrifugation. The proteome of the MV preparations was determined by LC-MS/MS analysis of peptides released from the MV by trypsin digestion. Both the number of unique peptide sequences (# peptides) and the total number of peptide to spectrum matches (#PSM) are displayed. Triplicate analysis were performed.

Annotation	**Accession ID[$]**	**Domains; additional annotation**	**Mw** (kDa)	**Predicted localization[€]**	**MV ***			
					DSM 17938		**BG-R46**	
					# peptides	# PSM	# Peptides	# PSM
Adhesion and cell interactions								
5'-nucleotidase	HMPREF0538_20056; Lr1025	LPXTG anchor; 5'-nucleotidase	80.7	Cell surface, LPxTG	25±1	54±10	17±5	23±5

LPXTG cell wall anchor domain-containing protein	HMPREF0538_20063; Lr1487	LPXTG anchor; 4x Coiled coil; putative type IV pilus biogenesis protein PilP; Rib/alpha-like repeat	68.3	Cell surface, LPxTG	17±2	19±3	8±1	8±1
MucBP protein	HMPREF0538_20356; Lr1612	KxYKxGKxW signal peptide; 5x MucBP motifs	172.3	Cell surface, GW	32±6	55±4	14±3	16±2
YSIRK-type signal peptide-containing protein	HMPREF0538_20775 HMPREF0538_20774; Lr1694	YSIRK signal; 9x Rib_alpha; putative adhesion protein; LPXTG anchor	187.8	Cell surface, LPxTG	21±1	38±2	22±2	34±3
Moonlighting proteins								
Elongation factor G	HMPREF0538_20596	Adhesin, binds salivary mucin MUC7	76.7	Cytoplasm	24±3	37±5	21±1	28±1
Phosphoketolase	HMPREF0538_20863	Mucin binding	91.4	Cytoplasm	30±6	64±1	25±2	39±2
6-Phosphogluconate dehydrogenase	HMPREF0538_20952	Adhesin	53.4	Cytoplasm	26±5	86±6	27±2	86±5
Aminopeptidase PepN	HMPREF0538_21097	Surface located peptidase[1]	95.3	Cytoplasm	35±5	112±26	26±6	42±8
ABC transporter, substrate-binding protein	HMPREF0538_21501; Lr0793	Collagen/Mucus binding protein CnBP [2]	28.5	Cell surface, pI >9	27±3	91±32	21±3	46±6
60 kDa chaperonin	HMPREF0538_21561	GroEL; binds mucins, epithelial cells and invertase	57.1	Cytoplasm	33±3	76±14	22±2	33±3
Glyceraldehyde-3-phosphate dehydrogenase	HMPREF0538_21606	Mucus- and cell-binding protein	37.0	Cytoplasm	20±1	286±129	19±2	166±30
Phosphoglycerate kinase	HMPREF0538_21607	Mucin binding	43.0	Cytoplasm	33±4	97±25	26±1	57±5
Enolase	HMPREF0538_21609	Fibronectin/plasminogen/ laminin binding	49.9	Cytoplasm	19±1	96±28	19±2	58±5
Pyruvate kinase	HMPREF0538_22005	Invertase binding	51.8	Cytoplasm	25±2	73±16	24±2	49±3

Glucose 6-phosphate isomerase	HMPREF0538_21641	Laminin and collagen binding	50.4	Cytoplasm	21±3	80±21	20±2	38±4
EPS production								
Peptidase, M23 family	HMPREF0538_20383; Lr0899	3x Cell wall-binding repeats (CW); Peptidase_M23; carbohydrate transport	101.1	Cell surface, GW	30±2	94±32	21±1	34±3
Dextran sucrase	HMPREF0538_20764; Lr1943	KxYKxGKxW signal peptide; glycosyl hydrolase family 70; 5x Cell wall-binding repeats (CW);	200.3	Cell surface, GW	97±9	259±61	59±11	79±13
Cell wall modulation								
Penicillin-binding protein Pbp2b, transpeptidase	HMPREF0538_20221; Lr0889	Transpeptidase (cell wall biosynthesis)	76.0	Membrane, cell surface	30±1	101±46	28±1	54±4
N-acetylmuramoyl-L-alanine amidase	HMPREF0538_20363; Lr1039	KxYKxGKxW signal peptide; Amidase domain	96.5	Cell surface, GW	24±4	34±3	9±3	10±3
Peptidase	HMPREF0538_20382; Lr0898	KxYKxGKxW signal peptide; Peptidase_C39-like	93.2	Cell surface, GW	22±4	34±1	8±2	9±3
Transferase	HMPREF0538_21056; Lr1829	Cell wall biogenesis	51.4	Membrane, cell surface	24±2	46±10	19±2	29±2
Peptidoglycan hydrolase	HMPREF0538_21064; Lr1822	KxYKxGKxW signal peptide; Glucosaminidase; 4x LysM;	60.5	Cell surface, LysM	29±3	164±64	30±1	134±33
D-alanyl-lipoteichoic acid biosynthesis protein DltD	HMPREF0538_21428; Lr1649	D-alanyl-lipoteichoic acid biosynthesis protein	49.4	Cell surface, Lipoprotein	20±4	58±7	17±2	39±1

DD-transpeptidase, Pbp1a	HMPREF0538_22189; Lr0545	Transpeptidase (cell wall biosynthesis)	81.9	Membrane, cell surface	32±4	135±40	28±3	52±5
Other membrane proteins								
Cation transport ATPase; MraZ	HMPREF0538_20821	Cation ATPase	100.1	Membrane	36±4	84±12	35±1	67±6
ABC transporter, ATP-binding protein	HMPREF0538_21067		71.8	Membrane	27±2	62±7	23±1	45±11
Uncharacterized surface protein	HMPREF0538_21191; Lr1863	C-terminal membrane anchor; 3x Coiled coil	103.6	Membrane, cell surface	26±2	36±2	29±1	48±7
Protein translocase subunit SecA	HMPREF0538_21575		90.4	Membrane	27±6	37±5	23±2	34±4
ATP synthase subunit alpha	HMPREF0538_21685	Proton-transporting ATP synthase activity	55.2	Membrane	30±1	189±68	30±2	176±29

$ Uniprot identity, https://www.uniprot.org/; Identity in Båth et al., 2005. Moonlighting protein identity, http://www.moonlightingproteins.org.

€ Predicted by analysis with SignalP and TMHMM; Cell surface localization according to Båth et al., 2005. Raw data file sorted after mean # of peptides in DSM 17938 MV. (1) Marquart, M.E., Pathogenicity and virulence of Streptococcus pneumoniae: Cutting to the chase on proteases. Virulence, 2021. **12**(1): p. 766-787.

* Average number of hits ± S.D.

Acta Universitatis Agriculturae Sueciae

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Ingestion of *Bifidobacterium longum* and *Limosilactobacillus reuteri* has known health-promoting effects. This thesis examined how probiotic bacteria interact with each other and with the host, and how production of probiotics can be manipulated to increase biological functionality. *L. reuteri* DSM 17938 and BG-R46 derived membrane vesicles and DSM 17938 derived exopolysaccharides were shown to elicit bioactive functions in models for host interactions. BG-L47, a novel strain of *B. longum* was characterized and shown to boost *L. reuteri* DSM 17938.

Ludwig Ermann Lundberg received his graduate education at the Department of Molecular Sciences, SLU, Uppsala. He received his Master of Science degree at Linköping University (One Year).

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