THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Systems-level characterization of probiotic bifidobacteria

Towards rational optimization of industrial production

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Cover: Illustration of the pleomorphic morphology of bifidobacteria. Besides the variability in their morphology, strains of the genus exhibit diverse stress physiologies, as discussed in this thesis.

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Nothing in life is to be feared. It is only to be understood.
— Marie Skłodowska-Curie

Preface

This doctoral thesis serves as partial fulfillment of the requirements for obtaining the PhD degree at the Division of Industrial Biotechnology at the Department of Biology and Biological Engineering at Chalmers University of Technology. The PhD studies were carried out between February 2018 and August 2022 under the supervision of Prof. Carl Johan Franzén. The thesis was examined by Prof. Lisbeth Olsson. The work was funded by Innovation Fund Denmark (grant no. 7038-00094A) and Chr. Hansen A/S.

Most of the work presented in this thesis was carried out at the Systems Biology Department, Discovery, R&D at Chr. Hansen A/S under the supervision of Dr. Ahmad A. Zeidan and under co-supervision of Dr. Anisha Goel. Cultivation experiments in 200 mL scale were conducted at the Upscaling Department, R&D at Chr. Hansen A/S. Biochemical analyses were performed at the Systems Biology Department and at the Biochemical Assays Department at Chr. Hansen A/S, as well as at the Division of Industrial Biotechnology and Chalmers Mass Spectrometry Infrastructure at Chalmers University of Technology.

Marie Schöpping
August, 2022

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Abstract

Probiotic *Bifidobacterium* strains contribute to a healthy gut microbiota of their hosts. Increasing public awareness of this positive effect has resulted in a growing demand for these microorganisms. During industrial production, probiotic microorganisms encounter environmental stressors, which can negatively impact their viability and health-promoting benefits. In this thesis, the current state of knowledge on robustness, stability, and stress physiology in bifidobacteria is reviewed, and the robust and stable *Bifidobacterium animalis* subsp. *lactis* BB-12® and the more sensitive *Bifidobacterium longum* subsp. *longum* BB-46 are investigated in detail.

The aim of this thesis was to compare the metabolism and physiology of BB-12® and BB-46, and to identify key determinants of growth and viability. The applied approach relied on the integration of constraint-based modeling, classical physiological analyses, and omics analyses. Strain-specific, thoroughly curated, genome-scale metabolic models were built for BB-12® and BB-46, and were applied to identity their nutritional requirements. This allowed for the formulation of a chemically defined medium that supported growth of both strains. The models and medium are valuable tools for optimizing industrial production of these two strains. BB-12® and BB-46 were studied in lab-scale cultivations in the newly formulated medium to identify correlations between cellular characteristics, robustness, and stability of bifidobacteria. Transcriptomic analysis revealed consistently higher expression of several stress-associated genes (e.g., chaperones) in BB-12® as compared to BB-46, which may explain the higher stress tolerance of BB-12®. Upregulation of genes related to DNA repair in BB-46 coincided with increased robustness and stability in stationary compared to exponential phase. The composition of the cultivation medium had a considerable impact on growth and stability of BB-12® and BB-46. The cell membrane fatty acid profile was identified as a key determinant of robustness and stability, by omitting Tween® 80 from the medium. An unsaturated to saturated fatty acid ratio below or around one was found to be beneficial. Moreover, a complex nitrogen source was found to reduce the survival of BB-46, and an increased cell size of BB-12® in complex MRS medium was proposed to contribute to its poor survival under this condition. To assess for possible correlations between gene content and the strain physiology under stress conditions, the genomes of 171 *Bifidobacterium* strains, including BB-12® and BB-46, were screened for the presence of known stress-associated genes, resulting in the postulation of putative genotype-phenotype correlations. The long-term objective is to use the knowledge gained in this work to guide rational optimization of industrial production processes involving probiotic bifidobacteria.

Keywords: bifidobacteria, probiotics, industrial manufacturing, genome-scale metabolic modeling, nutritional requirement, robustness, stability, stress-associated genes, interspecies variations.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Schöpping M.**, Gaspar P., Neves A. R., Franzén C. J., and Zeidan A. A. (2021). Identifying the essential nutritional requirements of the probiotic bacteria *Bifidobacterium animalis* and *Bifidobacterium longum* through genome-scale modeling. *npj Systems Biology and Applications*. 7 (47). 1–15. DOI: 10.1038/s41540-021-00207-4.

II **Schöpping M.**, Zeidan A. A., and Franzén C. J. (2022). Stress responses in bifidobacteria. *Microbiology and Molecular Biology Reviews, Accepted for publication.*

III **Schöpping M.**, Goel A., Jensen K., Faria R. A., Franzén C. J., and Zeidan A. A. Novel insights into the molecular mechanisms underlying robustness and stability in probiotic bifidobacteria. *Submitted*.

IV **Schöpping M.**, Goel A., Franzén C. J., and Zeidan A. A. Medium composition affects robustness and stability of *Bifidobacterium longum* BB-46 and *Bifidobacterium animalis* BB-12. *Manuscript*.

V **Schöpping M.**, Vesth T., Jensen K., Franzén C. J., and Zeidan A. A. (2022). Genome-wide assessment of stress-associated genes in bifidobacteria. *Applied and Environmental Microbiology.* 7 (88). e0225121. DOI: 10.1128/aem.02251-21.

Contribution summary

I I contributed to the conceptualization and planning of the work, as well as to the development of relevant experimental protocols. I curated the model of BB-12®, as well as built and curated the model of BB-46. I performed most of the experiments, data analyses, model simulations, and results interpretation. I wrote most of the manuscript.

II I planned the work, collected, evaluated, summarized the literature, and wrote the original manuscript.

III I participated in the conceptualization and planning of the work. I developed most of the experimental protocols, carried out the experiments and data analysis, did most of the results interpretation, and wrote the original manuscript.

IV I contributed to the conceptualization and planning of the work. I performed the experiments, data analysis, results interpretation, and wrote the original manuscript.

V I contributed to the conceptualization of the work, carried out the data analysis and results interpretation and wrote the original manuscript.

Poster presentations

1. **Schöpping M.**, Goel A., Olsson L., Franzén C. J., and Zeidan, A. A. Rational improvement of biomass yield and stability of probiotic bifidobacteria – A systems biology approach. March 2018. SysBio2018. Innsbruck, Austria. Awarded with the SysBio2018 Award.

2. **Schöpping M.**, Franzén C. J., and Zeidan A. A. Application of genome-scale metabolic modeling to identify the nutritional requirements of two industrially relevant *Bifidobacterium* strains. July 2019. EMBO|EMBL Symposium: New Approaches and Concepts in Microbiology. Heidelberg, Germany.

3. **Schöpping M.**, Goel A., Franzén C. J., Jensen K., and Zeidan A. A. Why are you stressed? A systems-level comparison of two *Bifidobacterium* strains with different stability characteristics. August 2021. 13th Symposium on Lactic Acid Bacteria. Online. Awarded with the LABIP International Science Award 2021.

Contents

Abbreviations

CDM	Chemically defined medium
CDW	Cell dry weight
CFU	Colony forming units
CO_2	Carbon dioxide
BCP	Bacterioferritin comigratory protein
BiGG	Biochemical Genetic and Genomic
BOF	Biomass objective function
BSH	Bile salt hydrolase
Dps	DNA-binding protein from starved cells
FAS	Fatty acid synthase
FBA	Fructose-bisphosphate aldolase
G6PDH	Glucose-6-phosphate dehydrogenase
GEM	Genome-scale metabolic model
GENRE	Genome-scale network reconstruction
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HCl	Hydrochloric acid
H^+-TH	$NAD(P)^+$ transhydrogenase
$HO^{\cdot}$	Hydroxyl radical
KEGG	Kyoto Encyclopedia of Genes and Genomes
MRS	De Man, Rogosa and Sharpe
MsrAB	(Peptide)-L-methionine-(R/S) sulfoxide reductase
N_2	Nitrogen
NPOX	H_2O_2-forming NADPH oxidase
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
OD_{600}	Optical density at 600 nm
PEP	Phosphoenolpyruvate
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SNP	Single nucleotide polymorphism
TrxA	Thioredoxin
TrxR	Thioredoxin-disulfide reductase
UFA	Unsaturated fatty acids
Xfp	Xylulose-5-phosphate/fructose-6-phosphate phosphoketolase

1 Introduction

Bifidobacteria are widely used as probiotics due to their beneficial impact on health. During production, probiotic bifidobacteria are exposed to different stressors, which can compromise their viability and, therefore their effectiveness. The ability to survive the exposure to such stressors varies across strains. The work presented in this thesis delivers new insights on the metabolism and physiology of industrially relevant *Bifidobacterium* strains, as well as the factors influencing their growth and viability. The generated knowledge paves the way for rational improvement of industrial processes employed in the production of probiotic bifidobacteria.

1.1 Background

The human body is inhabited by a diverse community of 10^{13} to 10^{14} microorganisms, the so-called microbiota (Gill et al., 2006). Most microorganisms that colonize the human body reside within the gastrointestinal tract. The estimated weight of the gut microbiome is around 1–2 kg (Forsythe & Kunze, 2013), and its composition has been shown to play a major role in human health (Lynch & Pedersen, 2016). A healthy gut microbiota is characterized by the predominance of beneficial bacteria, which coexist in a balanced community, and a very low proportion of inflammation-inducing bacteria (Iebba et al., 2016). The healthy gut can be perturbed by factors such as the intake of antibiotics, leading to an imbalance in the composition and function of intestinal microorganisms (Iebba et al., 2016; Lynch & Pedersen, 2016).

One potential intervention to stimulate a healthy gut is the administration of probiotics. Probiotics are viable microorganisms that, when administered in sufficient number, promote the health of their host (Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in, 2002). Probiotic products are associated with several health-benefits such as prevention of antibiotic-associated diarrhea (Ouwehand et al., 2002). Strains of the genera *Lactobacillus* and *Bifidobacterium* are the most common bacteria in probiotic applications (de Vrese & Schrezenmeir, 2008).

Bifidobacteria are fastidious microorganisms and the elevated sensitivity of many strains to environmental stressors, such as acid, heat, and oxygen (O_2), is a major challenge for their commercial suppliers. The optimal growth temperature for bifidobacteria ranges from 37°C to 41°C and their preferred growth pH lies between 6.5 and 7.0 (Biavati et al., 2000; Biavati & Mattarelli, 2015). Moreover, bifidobacteria are classified as anaerobes, and only a few strains show moderate or high O_2 tolerance (Kawasaki et al., 2006; Mättö et al., 2004; Simpson et al., 2005).

The low stress tolerance of bifidobacteria can severely impact survival, preventing the delivery of sufficient numbers of viable cells in the final probiotic product. Loss of viability throughout production and storage is associated not only with increased manufacturing costs, but also with potentially reduced efficacy of the probiotic product (Gueimonde & Sánchez, 2012). To compensate for this limitation, a surplus of cells (enumerated by colony forming units (CFU)) is commonly added to products (Fenster et al., 2019).

The sensitivity of bifidobacteria to environmental stressors varies among species and strains (Mättö et al., 2004; Simpson et al., 2005; Vernazza et al., 2006), making some strains more suitable than others for biotechnological applications. *Bifidobacterium animalis* subsp. *lactis* BB-12® (hereafter referred to as BB-12) is known for its good survival during manufacturing and shelf-life stability during shipping/storage. However, the molecular mechanisms endowing BB-12 with high robustness and stability compared to other *Bifidobacterium* strains remains poorly understood.

The intrinsic stability and robustness of probiotic strains are important parameter when selecting new probiotic strains for commercialization. In this thesis, these terms are defined as follows.

Robustness: *"Ability of a strain to sustain its functionality despite being exposed to perturbations" (**Paper II**).*

Stability: *"Ability of a strain to remain viable under given environmental conditions during storage" (**Paper II**).*

Optimization of manufacturing processes could maximize the robustness and stability of the produced culture. This may even allow the industrial production of clinically relevant *Bifidobacterium* strains, whose commercialization has been hampered by their elevated stress sensitivity.

In the past, production of probiotics has been successfully optimized through traditional one-factor-at-a-time or statistical approaches that compare phenotypic traits, such as specific growth rate, biomass yield, and stability, under different production conditions. However, such empirical approaches cannot reveal the molecular mechanisms underlying a given phenotype. Understanding the genetic and mechanistic factors that influence stress tolerance by bifidobacteria could advance industrial-scale production of probiotics, as summarized in Box 1 (adapted from **Paper II**).

Box 1. Opportunities based on exploiting new knowledge on the molecular mechanisms underlying robustness and stability in bifidobacteria.

- Optimization of cultivation media for probiotic strains, aimed at enhancing stress tolerance and improving survival throughout manufacturing and storage.
- Knowledge-driven selection of novel probiotic strains based on genetic and/or cellular characteristics that contribute to higher stress tolerance.
- Identification of biomarkers that can be used for monitoring (or evaluating) robustness and stability.
- Design of metabolic conditioning strategies, aimed at improving stress tolerance by probiotic strains.
- Targeted genome editing of probiotic strains for improved stress tolerance (note: application of genetically engineered probiotics is restricted in most countries).

1.2 Aim and scope of the thesis

The overall aim of this work was to generate comprehensive system-level knowledge of the metabolism and physiology of selected probiotic bifidobacteria, with a focus on the identification of key factors influencing their growth and viability. Knowledge gained in this work is meant to guide the rational improvement of industrial-scale production processes of probiotic bifidobacteria.

To achieve this aim, the metabolic and physiological characteristics of two industrially relevant probiotic *Bifidobacterium* strains that are known to differ in stability and robustness were investigated and compared in cultivation experiments. *Bifidobacterium animalis* subsp. *lactis* BB-12 was chosen to represent an intrinsically robust and stable strain, whereas *B. longum* subsp. *longum* BB-46 (hereafter referred to as BB-46) was chosen as a relatively sensitive strain.

Industrial-scale production of probiotic bifidobacteria comprises several steps, including cultivation, downstream processing, formulation, and storage. This thesis focuses on the cultivation step. This decision was based on the hypotheses that (i) knowledge-based modification of the cultivation process can enhance the robustness and stability of the cells and (ii) cells with enhanced robustness and stability after cultivation will most likely show higher survival throughout the subsequent manufacturing and storage steps.

As shown in Figure 1, the workflow during the PhD project was organized around the following secondary goals, whose achievement will contribute to the overall aim.

i. To reconstruct high-quality genome-scale metabolic models describing the metabolic capabilities of BB-12 and BB-46 (**Paper I**).
ii. To develop a chemically defined medium supporting reproducible growth of BB-12 and BB-46 using a model- and data-driven approach (**Paper I**).
iii. To identify the nutritional requirements of BB-12 and BB-46 (**Paper I**)
iv. To review existing literature on the stress response in bifidobacteria (**Paper II**).
v. To identify key differences in the metabolism and physiology of BB-12 and BB-46 (**Paper III, Paper IV**).
vi. To investigate the influence of medium composition on growth, robustness, and stability of BB-12 and BB-46 (**Paper IV**).
vii. To examine the genetic diversity of bifidobacteria, including BB-12 and BB-46, in terms of the prevalence of stress-associated genes (**Paper V**).

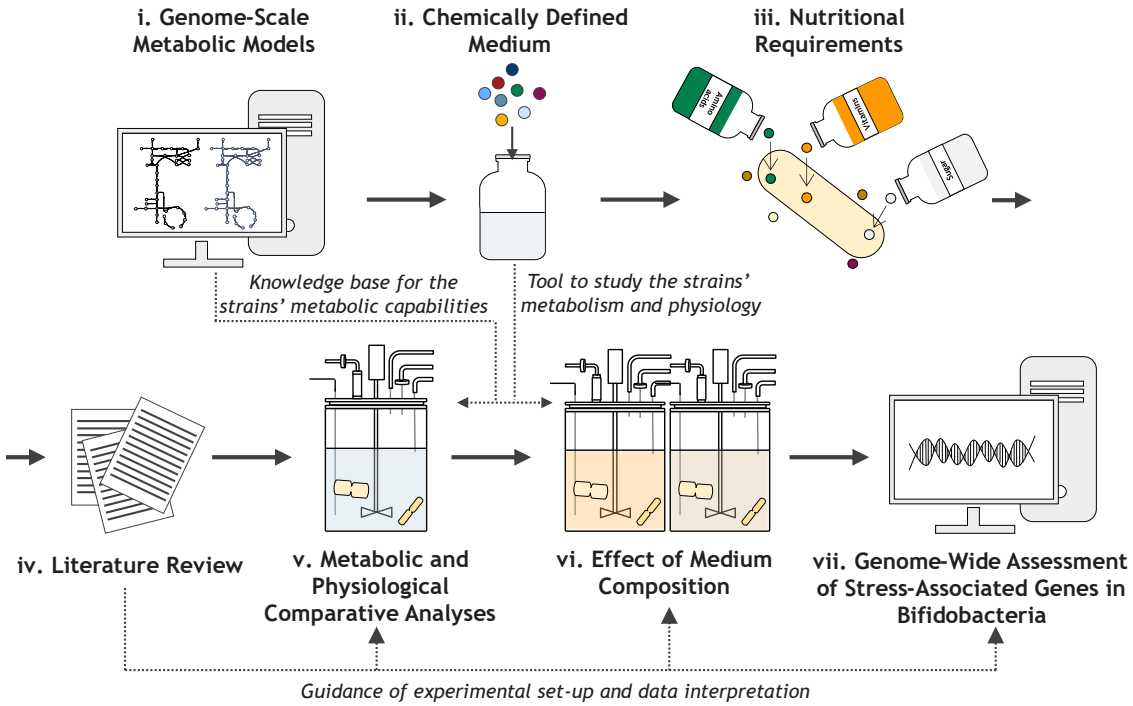


Figure 1: Workflow of the thesis. The work was organized to address the PhD project's secondary goals. Bold arrows illustrate the flow of the thesis, whereas dashed arrows show additional connections between its individual parts.

1.3 Structure of the thesis

The thesis is structured in six chapters. **Chapter 1** presents the background, as well as the aim and scope of thesis. A definition of probiotics and their health benefits, along with the use of bifidobacteria for this purpose are described in **Chapter 2**. In addition, this chapter offers an overview of industrial-scale production of probiotics and commonly encountered sources of stress associated with this process. The first section of **Chapter 3** is dedicated to the systems biology approach used in this work to study the metabolism of BB-12 and BB-46. In the following sections, the results of **Paper I** are presented and discussed in the context of existing knowledge. An overview of genome-scale metabolic models of bifidobacteria is provided, together with a summary of the central carbon metabolism, nutritional requirements, growth characteristics, and morphology of bifidobacteria. **Chapter 4** focuses on the molecular mechanisms underlying the varied stress response of bifidobacteria. First, the method used for assessing the robustness and stability of BB-12 and BB-46 is presented, and the rationale guiding this choice is explained. Subsequently, differences in stress tolerance among bifidobacteria are described. Previous knowledge on the stress response in bifidobacteria (**Paper II**) is discussed together with results from comparative studies on the metabolic and physiological characteristics of BB-12 and BB-46 (**Paper III, Paper IV**). In addition, the impact of medium composition on growth, robustness, and stability of BB-12 and BB-46 is discussed (**Paper IV**) and the results of a genomic study on the prevalence of stress-associated genes in bifidobacteria (**Paper V**) are presented. The chapter ends with an overview of genetic, metabolic, and physiological differences between BB-12 and BB-46 that have been identified in the course of the thesis, and which may determine the growth

behavior, robustness, and stability of the studied strains. In **Chapter 5,** the main findings of this work are summarized. In **Chapter 6**, an outlook on future research in the field is offered, focusing on how the results of the thesis can be used for the rational optimization of industrial production of bifidobacteria.

2 Probiotics

2.1 Definition and health benefits of probiotics

The foundation for the modern use of probiotics was laid more than a century ago, when Elie Metchnikoff proposed in 1907 that the consumption of lactobacilli in yoghurt had a beneficial effect on the health of Bulgarian people (Metchnikoff, 1907). Around 50 years later, the term *probiotics* (derived from the Greek and meaning 'for life') was introduced for the first time by Kollath to describe "organic and inorganic supplements necessary to restore health to patients suffering a form of malnutrition…" (Hamilton-Miller et al., 2003). Since then, the definition has been revised many times. In 1965, the term probiotic was applied in a slightly different context by Lilly and Stillwell to describe substances secreted by one microorganism, whose effect was to stimulate the growth of another (Lilly & Stillwell, 1965). A "substrate that is selectively utilized by host microorganisms conferring a health benefit" is nowadays denominated *prebiotic* (Gibson et al., 2017). In 1989, Roy Fuller defined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance", emphasizing for the first time the prerequisite of viability (Fuller, 1989). Nowadays, it is widely accepted that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in, 2002). This definition includes the need for viable probiotic microorganisms, as well as their application in a sufficient number.

Over the past decades, the interest in probiotics has increased tremendously, not least because of increasing knowledge of the human gut microbiome, emphasizing the central role of bacteria in human health. Various microorganisms, mainly strains of *Lactobacillus* and *Bifidobacterium* are commercially exploited in probiotic products (de Vrese & Schrezenmeir, 2008). The global probiotic market was estimated at 61.1 billion USD in 2021 and is predicted to grow to 91.1 billion USD by 2026 (www.marketsandmarkets.com, 24-05-2022).

When selecting microorganisms for application as probiotics, different criteria should be considered. First, probiotic strains must be sufficiently well characterized with respect to their strain identity (Binda et al., 2020; Huys et al., 2013). Second, they must be safe for consumption in food and dietary supplements (Binda et al., 2020; Huys et al., 2013). In the USA, probiotic microorganisms should be registered as Generally Recognized as Safe by the United States Food and Drug Administration, and in Europe they should have the Qualified Presumptions of Safety status by the European Food Safety Authority (Binda et al., 2020). Third, the health benefit of the strains in the target host must be documented in an appropriate trial (Binda et al., 2020; Huys et al., 2013). Fourth, in line with the definition of probiotics by the FAO/WHO, the strain must be viable in the probiotic product throughout its shelf life in a quantity that is sufficient to exert the claimed health benefit (Binda et al., 2020). This requirement is closely linked to the suitability of the probiotic strain for biotechnological purposes, and includes both its ability to survive all stages of industrial-scale processing and storage, as well as its sensitivity to various parameters over this time (Tripathi & Giri, 2014).

The effective dosage of a probiotic product depends on various factors, such as the type of strain(s) and the intended health effect (Bertazzoni et al., 2013). Therefore, the minimum quantity of cells that are required for a probiotic effect cannot be generalized. Currently available products recommend dosages between 10^9 to $4 \cdot 10^{10}$ CFU day^{-1}, although dosages at the lower end might not be efficacious (Bertazzoni et al., 2013).

Proposed health benefits of probiotic strains are multifold, including for example prevention or easing of diarrhea and prevention of respiratory tract infections (de Vrese & Schrezenmeir, 2008). The three main mechanisms of action of probiotics include:

i. **Inhibition of pathogens and toxins.** Pathogen inhibition by probiotics is attributed to the release of antimicrobial substances (e.g., organic acids), competition for nutrients and adhesion sites, removal and degradation of toxins, modulation of virulence and coaggregation with pathogens, as well as induction of immune responses in the host (Jungersen et al., 2014).
ii. **Enhanced intestinal barrier function.** The main components of the intestinal barrier are the outer mucus layer, populated by the gut microbiota, antimicrobial peptides, secretory immunoglobulin A molecules, intestinal epithelial cells, and the inner lamina propria, which contains various immune cells (Vancamelbeke & Vermeire, 2017). An intact intestinal barrier limits the transport of potentially harmful antigens and microorganisms from food and the environment (Rastall et al., 2005; Vancamelbeke & Vermeire, 2017). Probiotics are thought to strengthen this barrier by promoting the production of secretory immunoglobulin A molecules and mucus, as well as by facilitating proinflammatory responses (Rastall et al., 2005).
iii. **Modulation of the immune response.** Upon administration, probiotics induce specific and non-specific immune responses, including antibody responses, increased activity of natural killer cells and peripheral blood leukocytes, and enhanced cytokine production (Rastall et al., 2005).

Additional health benefits attributed to the administration of probiotic microorganisms have been reported, such as *in situ* production of B-vitamins, or the reduction of serum cholesterol (Nagpal et al., 2012; Ouwehand et al., 1999).

As stated before, the viability of probiotic strains is essential for their application. Nevertheless, some health benefits of probiotics do not seem to strictly depend on their viability. Instead, also non-viable cells and cell components, collectively termed as postbiotics or paraprobiotics, may benefit the host (Salminen et al., 2021; Siciliano et al., 2021). According to the International Scientific Association of Probiotics and Prebiotics, a postbiotic is defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). A probiotic cell that has lost viability during the shelf life of a probiotic product does not automatically fulfill this requirement (Vinderola, 2021). The health-promoting effects of a postbiotic seem to be influenced by the procedure used for its inactivation (de Almada et al., 2016) and the effectiveness and functionality of a postbiotic can differ from that of its viable counterpart (Castro-Herrera et al., 2020; Galdeano & Perdigón, 2004; Gill & Rutherfurd, 2001; Ouwehand & Salminen, 1998; Pyclik et al., 2021). In this thesis, the focus lies solely on the probiotic use of bifidobacteria in their viable form.

2.2 Bifidobacteria as probiotics

Bifidobacteria are Gram-positive, non-motile, non-spore forming bacteria with high G+C content that belong to the *Bifidobacteriaceae* family of the phylum *Actinobacteria*. They have been isolated from diverse niches, including the gastrointestinal tract, oral cavity, and vagina of humans, gastrointestinal tract of animals (mammals and insects), sewage, blood, and fermented food (Mattarelli & Biavati, 2018). So far, 54 different *Bifidobacterium* species have been documented (Mattarelli & Biavati, 2018). When it comes to their prevalence in the human gastrointestinal tract, it has been shown that *Bifidobacterium* strains dominate the microbiota of infants (60%–70%), but their relative quantity tend to decrease with age, amounting to only 2%–14% in adults (Arboleya et al., 2016).

Due to the high sensitivity of several strains to environmental stressors, the use of *Bifidobacterium* strains as probiotics is considered challenging. Today, five different *Bifidobacterium* species, including *B. animalis*, *B. longum*, *B. breve*, *B. bifidum*, and *B. adolescentis* are being commercially exploited in probiotic products (de Vrese & Schrezenmeir, 2008). All five of them have been granted a Quality Presumptions of Safety status (EFSA BIOHAZ Panel, 2022) and around 20 strains of these species are recognized as Generally Recognized as Safe (*GRAS Notices*, 2022). In particular, the Chr. Hansen strain BB-12 has been characterized in more than 300 scientific publications (Jungersen et al., 2014) and its annual production has reached around 175 tons.

2.3 Industrial-scale production of probiotics

The industrial-scale production of probiotic microorganisms, such as bifidobacteria, includes several steps (Figure 2) that need to be optimized to ensure consistent delivery of viable and functional probiotics. The first step in the production process is the preparation of a suitable cultivation medium. Often, industrial media are undefined as they contain numerous complex ingredients such as yeast extract (Stanbury et al., 2013). Yeast extract, for example, which serves as a carbon and nitrogen source, is composed of various amino acids, peptides, vitamins, growth factors, trace elements, and carbohydrates, whose exact quantity varies across batches and suppliers. This variation may mask small improvements in process productivity (Stanbury et al., 2013). Knowledge of the nutritional requirements of probiotic strains is essential for the formulation of media that ensure consistent high cell densities (Fenster et al., 2019). In this thesis, the application of genome-scale metabolic modeling (section 3.2), was shown to be a valuable tool for the design of growth medium for bifidobacteria (**Paper I**). The composition of cultivation medium does not only determine growth but can also affect the robustness and stability of the produced cells (**Paper IV**). This aspect is further described in section 4.4.

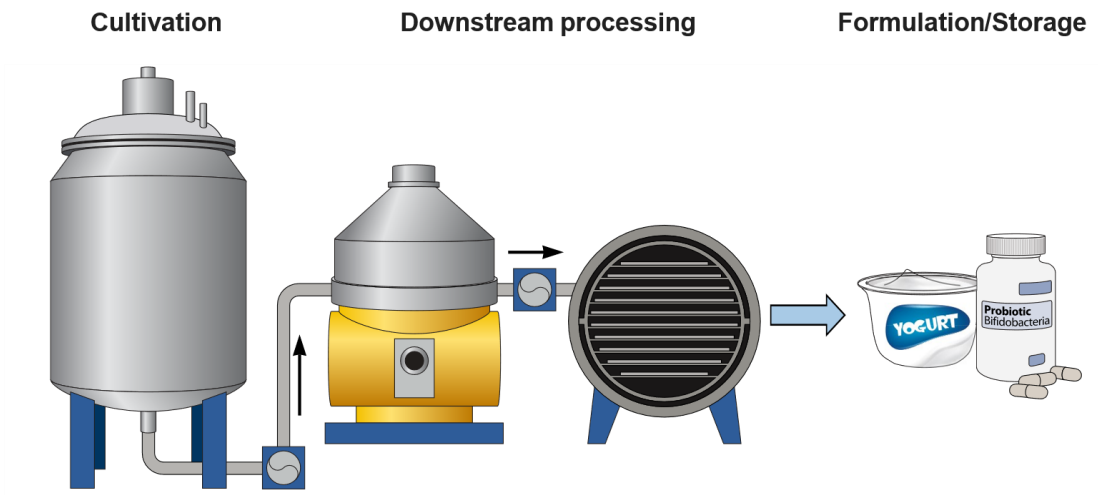


Figure 2: Simplified scheme summarizing the industrial-scale production of probiotics. Probiotic microorganisms are multiplied during the cultivation step, subjected to downstream processing, stored and finally included in pharmaceutical or food products, before being consumed (adapted from Figure 1 in **Paper II**).

To ensure reproducible performance and quality of the produced cells, bioprocess parameters, such as pH, temperature, and dissolved oxygen, should be tightly controlled (Høier et al., 2010). In general, industrial cultivation processes aim for a high product yield, defined by the amount of product (cells) per amount of substrate (carbohydrate) used (kg/kg), and a high productivity, defined by the amount of product (cells) generated per time unit (kg/h), which relate to the biomass yield and specific growth rate if biomass is the product. In the case of probiotics, it is the number of viable cells produced rather than their absolute amount that represents the main goal. Additional factors include robustness and stability of the produced cells, as they will determine the proportion of cells that lose viability during downstream processing, as well as the surplus required to ensure that minimum effective number of cells at the time of administration. Both these parameters directly affect the costs and resource efficiency during production.

After the cultivation step, the cells are subjected to downstream processing (Figure 2), whereby they are separated from the cultivation broth by centrifugation or membrane filtration, prior to long-term preservation treatment (Høier et al., 2010; Modesto, 2018) to minimize the effect of storage on cell viability and functionality (Modesto, 2018). Different methods can be used for long-term preservation, including freezing (cryopreservation), freeze-drying (lyophilization), spray-drying, vacuum-drying, air-drying and fluidized-bed drying (Modesto, 2018). In case the preservation treatment includes freezing, the concentrated probiotic culture is mixed with a cryoprotectant in order to shield the cells from freezing stress (Hubálek, 2003; Tripathi & Giri, 2014). The choice of preservation method depends on the final application of the probiotic culture, but freezing and freeze-drying are the most common choices. Freezing of the probiotic culture can be carried out by dropping concentrated culture into liquid nitrogen (-196°C), resulting in the formation of frozen cell pellets (Høier et al., 2010). Drying extends the shelf-life of the cells at ambient temperature, which is a considerable advantage for transportation, storage, and application in functional foods (Modesto, 2018; Tripathi & Giri, 2014). Freeze-dried material can be ground into

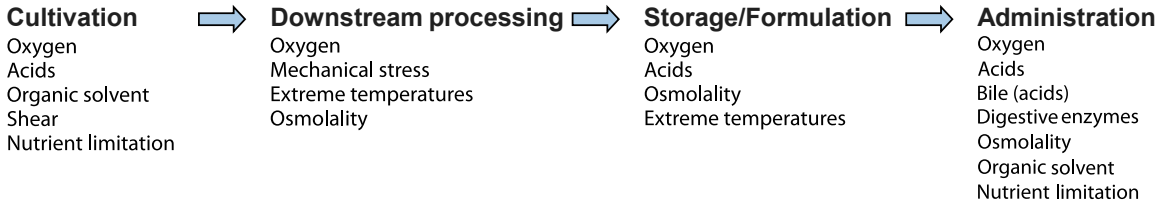


Figure 3: Environmental stressors present at different stages during the lifespan of a probiotic microorganism, from cultivation (production) to administration. At each stage, different stressors become dominant and can affect the viability of probiotic strains (adapted from Figure 1 in **Paper II**).

small particles, blended with excipients that are optimized for the final application, and then used to formulate the target probiotic product (Figure 2) (Fenster et al., 2019). Probiotic cultures are commonly supplied to the customer as dietary supplements (e.g., capsules) or incorporated into fermented (e.g., whey-based drinks, cheese) and non-fermented food products (e.g., sweets, fruit juices) (de Vrese & Schrezenmeir, 2008; Tripathi & Giri, 2014). The quality of the probiotic culture is tested at different steps along the manufacturing process.

2.4 Stressors commonly encountered during the lifespan of probiotics

Over their lifespan, commercially used probiotic microorganisms encounter various environmental stressors, which can lead to loss of viability and functionality (Amund, 2016; Fenster et al., 2019; Lacroix & Yildirim, 2007; Tripathi & Giri, 2014). The prevailing stressors are O_2, heat, cold, high osmolality, nutrient limitations, organic solvents, mechanical forces, organic acids, as well as stressors in the gastrointestinal tract, such as bile (acids) and digestive enzymes (Figure 3).

During cultivation, the prevailing stressors include organic acids and organic solvent (ethanol), which are formed as end products of microbial fermentation. An additional concern is oxidative stress, whose potentially toxic effect on cell physiology is minimized by removing O_2 from the broth upon continuous flushing of the headspace of the reactors. Even though process parameters, such as pH and dissolved oxygen, are rigidly controlled during cultivation to minimize perturbations, large time constants typical of industrial scale production may cause the formation of gradients, which expose the cells to suboptimal conditions. Moreover, not all perturbations can be avoided, and the cultivation process might be impaired e.g., by variations in raw material, equipment failure, or the implementation of new processes and equipment (Fenster et al., 2019).

After cultivation, probiotic strains are exposed to heat and shear stress during centrifugation (Fenster et al., 2019). Any additional stressors during downstream processing depend on the choice of preservation treatment. For example, during freezing and freeze-drying, cells are exposed to a cold shock; whereas spray-drying exposes them to a heat shock (Tripathi & Giri, 2014). Further adverse events during drying include osmotic and mechanical stress (Tripathi & Giri, 2014).

During shelf-life as frozen or dried products or as ingredients in a food product, the viability of probiotic strains can be affected by various parameters, including water activity, storage temperature, pH, and O_2 content (Abe et al., 2009; Celik & O'Sullivan, 2013; Fenster et al., 2019; Lacroix & Yildirim, 2007; Tripathi & Giri, 2014). When incorporated in food products, the survival of probiotic strains depends also on food processing conditions (Nagpal et al., 2012; Tripathi & Giri, 2014).

Upon administration, the probiotic strains are challenged by stressors in the gastrointestinal tract of the host, such as O_2 (in the oral cavity and stomach), acids (HCl in the stomach, bile acids in the small intestine), and changes in nutrient availability (Lacroix & Yildirim, 2007; Ruiz et al., 2011).

The ability of *Bifidobacterium* strains to resist the dominant stressors during manufacturing, storage, and administration varies considerably among species and strains, as further discussed in section 4.2 (Celik & O'Sullivan, 2013; Mättö et al., 2004; Modesto et al., 2004; Vernazza et al., 2006). The molecular basis of such variability in robustness and stability of bifidobacteria was the focus of this thesis.

2.5 Summary

Bifidobacteria are used as probiotics to benefit the host's health. They act by inhibiting pathogens and toxins, promoting the intestinal barrier function, and modulating the immune response. The first step during industrial production of probiotics is the cultivation, followed by downstream processing, formulation, and storage. Generally, probiotics must be viable at the time of administration. However, during manufacturing, probiotic bifidobacteria are subjected to numerous stressors, which can lead to loss of viability. The sensitivity towards these stressors varies across *Bifidobacterium* strains and the molecular mechanism underlying this variation has not been fully understood.

3 Metabolism and physiology of bifidobacteria

Extensive research on the health benefits of probiotic bifidobacteria over the last decades has not been matched by an equal interest in their metabolism and physiology. For example, the regulation of carbohydrate dissimilation, and the molecular mechanisms underlying the stress physiology of bifidobacteria remain poorly understood. Improved understanding of the metabolic and physiological characteristics of bifidobacteria could enable the rational design of strategies aimed at maximizing growth, robustness, and stability of probiotic bifidobacteria during industrial production, resulting in a more effective commercial usage of these microorganisms.

3.1 Systems-level analysis of the metabolism and physiology of bifidobacteria

To study the metabolism and physiology of the two industrially and clinically relevant strains BB-12 and BB-46, a systems biology approach was applied in this work. Systems biology combines experimental and computational methods to study biological units in their entirety. The objective is to reveal the interplay between the cellular components of a system and to understand how the ensemble of all such components results in the observable phenotype (Palsson 2006). Systems biology is complementary but also interdependent to the methodological reductionist approach, which focuses on individual components of a complex system (Fang and Casadevall 2011). The dependency of systems biology on reductionism occurs because (i) it relies on mechanistic knowledge gained in reductionistic studies and (ii) it delivers hypotheses that may need to be confirmed by applying reductionistic approaches (Fang and Casadevall 2011; Nielsen 2017; Teusink et al., 2011).

To understand the cellular processes of a biological system in their entirety, detailed knowledge of their constituting biomolecules is required (Hein et al. 2013). High-throughput omics analyses are commonly applied to deliver system-level information on the chemical composition of a biological system, such as a bacterial cell. These omics analyses include: (i) genomics, i.e., genome sequencing and annotation, (ii) transcriptomics, i.e., the quantity of mRNA at a given condition, (iii) proteomics, i.e., the quantity of proteins, their interactions, and their functional state, (iv) metabolomics, i.e., the quantity of metabolites, and (v) flux-omics, i.e., metabolic fluxes (Palsson 2006). Omics analysis results in the generation of large and complex datasets that are difficult to interpret (Palsson and Zengler 2010). To cope with the elevated complexity of biological systems and big data, mathematical models are an indispensable tool in systems biology. Thus, constraint-based genome-scale metabolic modeling or kinetic models are commonly applied to study the metabolism of an organism (Nielsen 2017; Teusink et al., 2011).

Omics technology can be useful for the development of industrial-scale probiotics production as it can reveal strain-specific nutritional requirements and capabilities (Fenster et al. 2019). In the systems biology approach presented in this thesis, computational and experimental analyses have been combined to gain better understanding of the metabolism

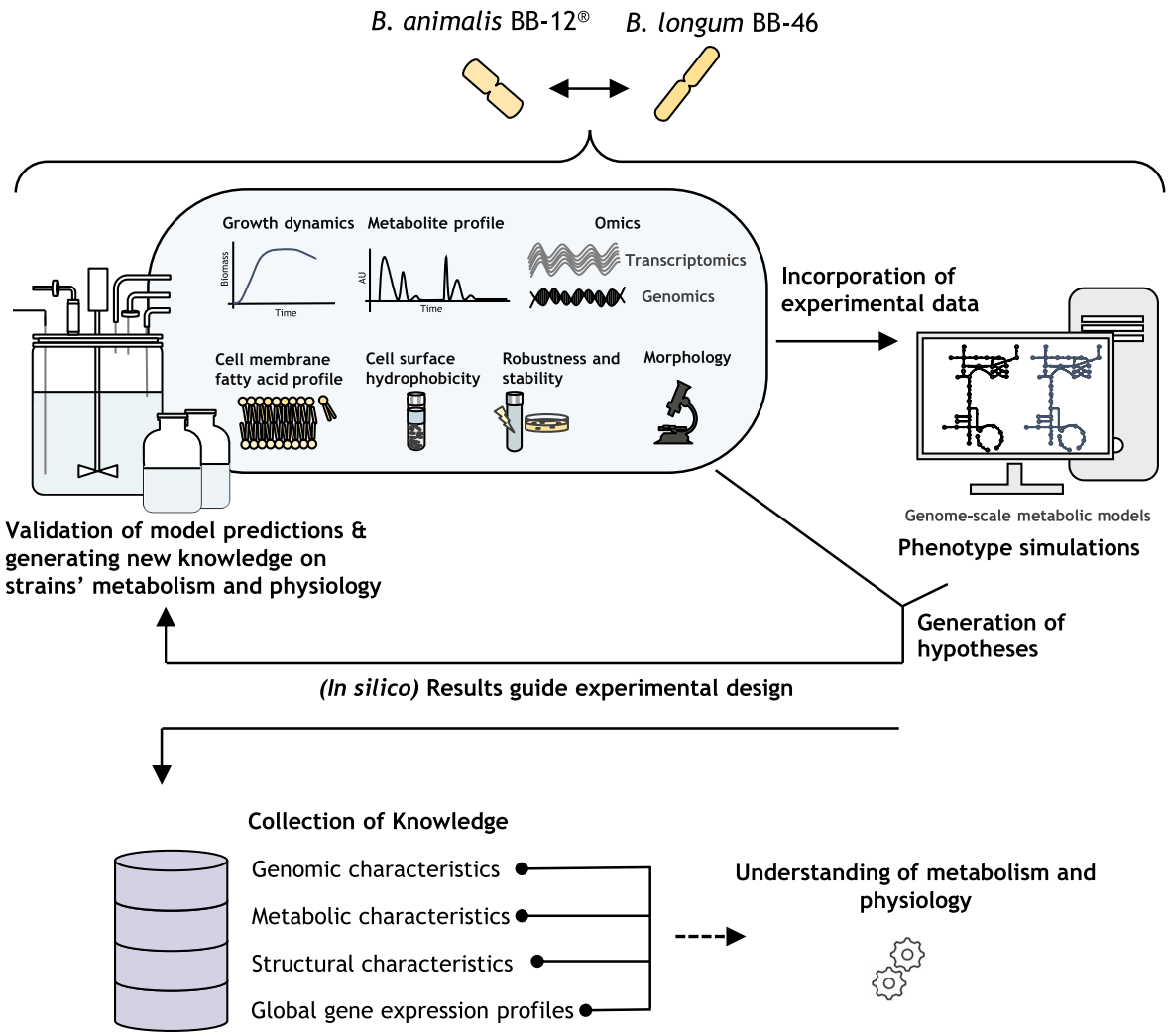


Figure 4: Systems biology approach applied in this thesis to study and compare the metabolism and physiology of BB-12 and BB-46. BB-12 and BB-46 were cultivated in batch mode, and their metabolism and physiology were studied via different *in vivo* analyses. The obtained information was analyzed and incorporated into newly developed genome-scale metabolic models of BB-12 and BB-46 that were applied for phenotype simulations. New experimental and *in silico* results allowed the formulation of hypotheses that guided the design of further experiments. Overall, this approach resulted in improved understanding of the metabolism and physiology of the tested strains.

and physiology of BB-12 and BB-46. This information can be used to optimize growth, robustness, and stability during industrial-scale production (Figure 4).

New experimental data on BB-12 and BB-46 were collected from batch cultivation experiments. To characterize the metabolism and physiology of BB-12 and BB-46 various *in vivo* analyses were carried out. Besides genomics (**Paper I, Paper IV, and Paper V**) and transcriptomics (**Paper III**), the two strains were studied in terms of their growth dynamics and metabolite profile (**Paper I, Paper III, Paper IV**), cell membrane fatty acid profile, as well as cell surface hydrophobicity and morphology (**Paper III, Paper IV**). The robustness and stability of the two strains were determined by assessing survival during short-term storage under different suboptimal conditions (**Paper III, Paper IV**). The method used for viability assessment, the conditions selected for the stress test, and the rationale behind their

selection are discussed in detail in section 4.1. The data collected from various experiments were analyzed and used to develop and refine strain-specific genome-scale metabolic models (GEMs) of BB-12 and BB-46. The models were used to run phenotype simulations and to explore the metabolism of the two strains. *In silico* results were coupled to experimental data to generate new hypotheses on the metabolism and physiology of BB-12 and BB-46. These hypotheses guided the design of experiments that aimed to validate the models' predictions and generate new key information on the strains' metabolism and physiology. By repeating this cycle, increasing genomic, metabolic, and structural knowledge on BB-12 and BB-46 was collected and used to explain the divergent stress physiology of the two strains (Figure 4). Details of the results of cultivation experiments, physiological analyses, and genome-scale metabolic modeling are presented in this and the following chapter.

3.2 Genome-scale metabolic models of bifidobacteria

Within the context of this work, strain-specific GEMs of BB-12 (hereafter referred to as *i*AZ480) and BB-46 (hereafter referred to as *i*MS520) were developed and subjected to qualitative and quantitative analysis of their metabolic capabilities (**Paper I**). The first version of the BB-12 GEM had been constructed before the initiation of this PhD project, but was curated within it.

A GEM is a popular tool in microbial systems biology. It is derived from a strain-specific genome-scale network reconstruction (GENRE) that lists all known metabolic reactions and genes of an organism based on (i) genome annotation, (ii) biochemical data, and (iii) physiological data (Thiele and Palsson 2010). In this work, the data were retrieved as follows.

i. Whereas the genome sequence of BB-12 had been published before and updated recently (Garrigues et al., 2010; Jensen et al., 2021), BB-46 was genome-sequenced during the course of this thesis. The genomes were structurally and functionally annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016) (**Paper I**).
ii. Biochemical data for the manual curation of *i*AZ480 and *i*MS520 were retrieved from online databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, 2000), TransportDB (Ren et al., 2007), and BioCyc (Karp et al., 2019) (**Paper I**).
iii. Physiological data were compiled from previous studies on BB-12 and BB-46 or closely related microorganisms, as well as from new experiments performed as part of this thesis (**Paper I**).

Each reaction in a GENRE is associated with the encoding gene(s), enzyme(s), and additional information, such as the reaction formula and directionality, resulting in a valuable knowledge base on the metabolic network of the organism (Thiele and Palsson 2010).

To generate a GEM, the reconstructed metabolic network is converted into a stochiometric matrix S. In the stoichiometric matrix every column represents a reaction and every row a metabolite, so that the entries in every column correspond to the stoichiometric coefficients for all metabolites in each reaction in the reconstruction (Thiele and Palsson 2010). When

used with constraint-based modeling tools such as flux balance analysis, GEMs can be used to simulate the metabolic state. This is represented by the fluxes through all metabolic reactions, which are commonly expressed in mmol g_{CDW}^{-1} h^{-1}, where CDW is the cell dry weight (Orth et al. 2010; Bordbar et al. 2014). The solution space of the constraint-based analysis defines all feasible flux distributions of the metabolic network (Bordbar et al. 2014). It is constrained by a steady state assumption whereby intracellular metabolites are neither consumed nor produced ($S \cdot v = 0$), resulting in a linear system of mass balance equations (Orth et al., 2010). Additional constraints can be imposed by directionality and measured fluxes such as sugar uptake rates (Bordbar et al. 2014). To measure metabolic fluxes of a microorganism at steady state, chemostat cultivations are normally set up. Alternatively, metabolic fluxes can be determined in batch cultivations, assuming a pseudo-steady state in the exponential growth phase, when nutrients are present in excess and strains grow at their maximum specific growth rate for the given condition. This latter approach was applied in this thesis (**Paper I**).

Despite the introduction of constraints, the system is usually underdetermined, i.e., there are too few equations for too many unknown fluxes. Therefore, programming is used to optimize (minimize/maximize) metabolic fluxes through the network for a selected objective function, commonly the biomass objective function (BOF) (Orth et al., 2010). The BOF describes the rate at which all metabolites that are required for biomass formation are synthesized in correct proportions, so that BOF and growth rate optimization coincide (Feist & Palsson, 2010).

As highlighted in **Paper I**, inclusion of the correct nutrients in the BOF is crucial when determining the nutritional requirements of a strain using flux balance analysis, as every nutrient must either be synthesized by the strain or supplied to it. Therefore, a *Bifidobacterium*-specific BOF for *i*AZ480 and *i*MS520 was formulated based on the macromolecular composition of BB-12, as well as data available on bifidobacteria and closely related strains (**Paper I**). For *i*MS520, the peptidoglycan composition in the BOF was adjusted to the cell wall type of *B. longum* strains (**Paper I**). The BOF was further curated based on experimental results (**Paper I**).

Due to the large size of metabolic networks, several flux distribution patterns may attain the same BOF value (Mahadevan and Schilling 2003). Flux balance analysis then selects one particular solution from the overall solution space that maximizes/minimizes the objective function (Orth et al., 2010).

Genome-scale metabolic reconstruction combined with constraint-based modeling is a powerful technique in systems biology as it links the wealth of information embedded in the DNA sequence of the genome with the phenotype of a strain (Rau and Zeidan 2018). Besides the use for phenotype simulations, GEMs can serve as a structured platform to facilitate the biological interpretation of experimental omics data (Rau and Zeidan 2018). The main disadvantage of genome-scale metabolic modeling is the inability to consider regulation or predict metabolite concentrations, because kinetic parameters are not incorporated (Orth et al., 2010).

Constructing a GEM from scratch is a time-consuming process (Thiele and Palsson 2010). Therefore, several automated tools for the reconstruction of draft networks have been

developed (Mendoza et al. 2019), including MetaDraft (Olivier 2019), and Model SEED (Henry et al. 2010). The utilization of manually curated GEMs of closely related strains as templates improves the quality of the automatically generated draft and accelerates the reconstruction process (Mendoza et al. 2019). Therefore, a template-based, semi-automated approach based on Model SEED and MetaDraft was applied to reconstruct the GEMs of BB-12 and BB-46 (**Paper I**).

Even though a few GEMs of *Bifidobacterium* strains have been published (Devika & Raman, 2019; El-Semman et al., 2014; Karp et al., 2018), they have been subjected to limited manual curation and/or experimental validation.

In 2014, the first GEM of a *Bifidobacterium* strain was that of *B. adolescentis* L2-32 (*i*Bif452) (El-Semman et al., 2014). *i*Bif452 was used to study the synergetic interaction between *B. adolescentis* L2-32 and *Faecalibacterium prausnitzii* A2-165, both of which populate the human gut microbiome (El-Semman et al., 2014). The model was constructed by applying a semi-automated approach based on the use of KEGG Orthology and KEGG pathways (El-Semman et al., 2014; Kanehisa, 2000). An inherent issue with this approach is that a reaction catalyzed by a multienzyme complex is added to the network reconstruction even if only one of the encoding genes is found in the genome of the strain. For example, *i*Bif452 includes the reaction of the multienzyme complex 2-oxoglutarate dehydrogenase solely due to the presence of a gene annotated as dihydrolipoamide dehydrogenase. In this work, during reconstruction of the GEMs of BB-12 and BB-46, special precaution was taken to validate the presence of the complete gene setup of multi-enzyme complexes, which resulted in the exclusion of 2-oxoglutarate dehydrogenase activity.

In 2018, a GEM of *B. longum* subsp. *longum* JCM 1217 was constructed using the Pathway Tool software of the BioCyc database (Karp et al., 2016, 2018). The resulting GEM was then used to compare the outcome of manual and automatic gap-filling of genome-scale metabolic model reconstructions (Karp et al., 2018).

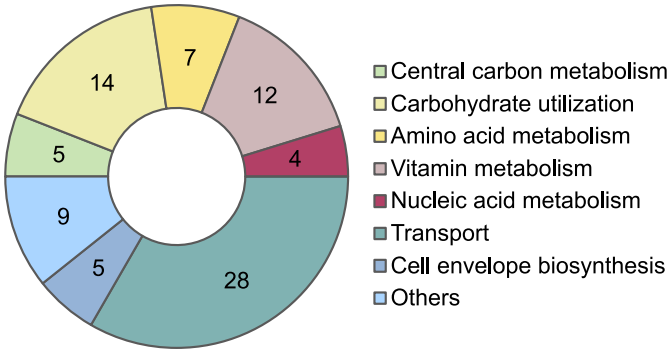
In 2019, GEMs of 36 *Bifidobacterium* strains covering 20 different species were used to predict the strains' metabolic capabilities (Devika & Raman, 2019). The GEMs were retrieved from Assembly of Gut Organisms through Reconstruction and Analysis, which is a source for semi-automatically generated GEMs of gut microorganisms (Devika & Raman, 2019; Magnúsdóttir et al., 2017). Prior to their use with constraint-based modeling, the retrieved GEMs were manually curated but only with respect to the predicted carbohydrate utilization profiles (Devika & Raman, 2019).

In contrast to previously published GEMs of *Bifidobacterium* strains, the GEMs of BB-12 and BB-46 were subjected to extensive manual curation and experimental validations in this work (**Paper I**). Moreover, as mentioned before, while previous GEMs contain simplified, non-genus-specific BOFs (El-Semman et al., 2014; Karp et al., 2018; Magnúsdóttir et al., 2017), *i*AZ480 and *i*MS520 include a *Bifidobacterium*-specific BOF, improving the prediction accuracy of the two GEMs. The characteristics of *i*AZ480 and *i*MS520 are summarized and compared with the properties of the first published model, *i*Bif452, in Figure 5.

A

Bifidobacterium strain	*B. animalis* BB-12 (*i*AZ480)	*B. longum* BB-46 (*i*MS520)	*B. adolescentis* L2-32[1] (*i*Bif452)
Genome size	1.9 Mb	2.4 Mb	2.4 Mb
Protein-encoding genes	1540	1870	2428
Genes included (% of protein-encoding genes)	480 (31%)	520 (28%)	452 (19%)
Reactions	731	771	699
Reactions (metabolic, transport)	639	677	640
Gene-associated	577	622	615
Non-gene associated	62	55	25
Exchange reactions	86	88	59
Demand/ sink reactions	4/2	4/2	0/0
Metabolites[2]	669	680	768

B **Reactions not shared between *i*AZ480 and *i*MS520, organized by subsystems**



C **Venn diagram of reactions in *i*AZ480, *i*MS520 and *i*Bif452**

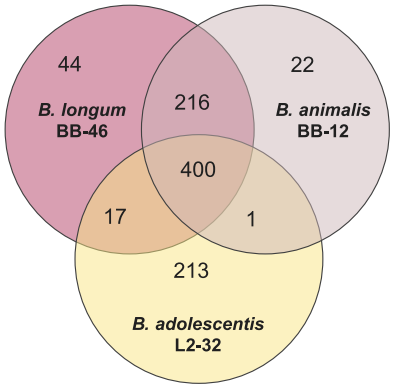


Figure 5: Comparison of the metabolic networks of *i*AZ480, *i*MS520, and *i*Bif452. A. Principal characteristics of *i*AZ480, *i*MS520, and *i*Bif452.[1] (El-Semman et al., 2014).[2] Metabolites are counted twice if they exist in both the cytoplasm and the extracellular matrix. **B**. Overview of the reactions that are not shared between *i*AZ480 and *i*MS520. **C.** Venn diagram of reactions included in *i*AZ480, *i*MS520, and *i*Bif452. The comparison was performed based on BiGG reaction identifiers and EC numbers (adapted from Figure in **Paper I** under the CC BY 4.0 license).

The capabilities of *i*AZ480 and *i*MS520 to predict known metabolic characteristics of bifidobacteria (section 3.3), the carbon utilization profile, and the vitamin and amino acid requirements of both strains were validated (section 3.4) in **Paper I**. The resulting information was applied to develop a chemically defined medium that supported growth of both strains (section 3.4). Moreover, growth and metabolite secretion rates predicted by the two GEMs were quantitatively validated in lab-scale batch cultivations (section 3.5). Finally, *i*AZ480 and *i*MS520 were used as comprehensive descriptors of the metabolic network of BB-12 and BB-46, and served for the analysis and interpretation of newly collected experimental data. Among others, this allowed the assignment of more precise functions to metabolic genes than those provided by functional annotation (**Paper III)**.

3.3 Central carbon metabolism of bifidobacteria

Reactions of the central metabolism of bifidobacteria have been included in *i*AZ480 and *i*MS520 based on genomics data, as well as physiological data available for bifidobacteria (**Paper I**).

Bifidobacteria lack a gene encoding 6-phosphofructokinase (EC 2.7.1.11) (Brandt & Barrangou, 2016; González-Rodríguez et al., 2013), and are therefore not able to dissimilate hexoses via the Embden-Meyerhof Parnas Pathway. Instead, they use a unique heterofermentative pathway known as the 'bifid shunt'. The key enzyme of this pathway is xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp; EC 4.1.2.22), which has dual substrate specificity for fructose-6-phosphate and xylulose-5-phosphate (Meile et al., 2001). In contrast, phosphoketolases (EC 4.1.2.9) from other genera have a strong preference for xylulose-5-phosphate only (Fushinobu, 2010). In the bifid shunt, Xfp converts one mole of fructose-6-phosphate into one mole of acetyl-phosphate and one mole of D-erythrose-4-phosphate (Figure 6) (González-Rodríguez et al., 2013). D-erythrose-4-phosphate can be converted into xylulose-5-phosphate by the successive action of transaldolase and transketolase (Figure 6). When acting on xylulose-5-phosphate, Xfp forms one mole of acetyl-phosphate and one mole of D-glyceraldehyde-3-phosphate (Figure 6). Acetyl-phosphate is further converted to acetate by acetate kinase, resulting in the formation of one ATP, whereas D-glyeraldehyde-3-phosphate is further metabolized into different fermentation end products (Figure 6).

Early studies reported that some *Bifidobacterium* strains lacked fructose-bisphosphate aldolase (FBA; EC 4.1.2.13) activity, catalyzing the reversible conversion of fructose-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Figure 6), and/or glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), catalyzing the oxidation of glucose-6-phosphate to 6-phospho-D-glucono-1,5-lactone (Figure 6) (De Vries & Stouthamer, 1967; Scardovi & Crociani, 1974). The reaction catalyzed by G6PDH represents the first step of the pentose phosphate pathway and is important for the generation of NADPH (Spaans et al., 2015). Based on the absence of FBA and G6PDH activity in some strains, both enzymes have been sometimes assumed to be generally absent from bifidobacteria (Leroy et al., 2013; Palframan et al., 2003). However, other studies have detected FBA and G6PDH activity in certain strains (Matteuzzi et al., 1971; Scardovi & Sgorbati, 1974; Scardovi & Trovatelli, 1974). In *B. longum* BBMN68, the gene encoding G6PDH was further found to be induced upon bile stress (An et al., 2014) and acid stress (Jin et al., 2012). Moreover, both BB-12 and BB-46 harbor a gene annotated as G6PDH (Figure 6). Metabolic flux through the G6PDH-mediated phosphate pathway would not only contribute to NADPH generation, but it would also represent an alternative route to feed Xfp with xylulose-5-phosphate (An et al., 2014) (Figure 6).

The essentiality of G6PDH activity for a functional metabolism in BB-12 and BB-46 was tested using *i*AZ480 and *i*MS520. The GEMs predicted an absolute requirement for G6PDH activity unless NADPH could be generated via proton-translocating, membrane-bound $NAD(P)^+$ transhydrogenase activity (H^+-TH; EC 1.6.1.2) (unpublished data). *Bifidobacterium* strains devoid of G6PDH activity might thus use H^+-TH for NADPH generation. A recent study has confirmed the presence of genes annotated as H^+-TH in nine

Bifidobacterium strains of different species, including *B. animalis* and *B. longum*, but without testing for enzymatic activity (Zafar & Saier Jr., 2021). Consistent with these results, both BB-12 and BB-46 possess genes encoding H^+-TH. However, due to incorrect assignment of the EC number 1.6.1.2 to the reaction catalyzed by the energy-independent soluble transhydrogenase (STH; EC 1.6.1.1) in the Biochemical Genetic and Genomic (BiGG) database (King et al., 2016), the reaction equation in *i*AZ480 and *i*MS520 corresponds to that of STH and will need to be corrected. The measured FBA activity in *Bifidobacterium* strains is generally low and its function in bifidobacterial carbon metabolism remains unknown (Scardovi & Crociani, 1974; Scardovi & Sgorbati, 1974).

As mentioned above, D-glyeraldehyde-3-phosphate generated by Xfp in the bifid shunt can be converted to different fermentation end products (Figure 6). Theoretically, the dissimilation of two moles of glucose via the bifid shunt leads to the formation of two moles of lactate and three moles of acetate, resulting in 2.5 ATP per mole of hexose catabolized (Palframan et al., 2003). In practice, formate, ethanol, and succinate have also been detected as fermentation end products of bifidobacteria (**Paper I, Paper III, Paper IV**) (Amaretti et al., 2007; De Vries & Stouthamer, 1968; Degnan & Macfarlane, 1994; González-Rodríguez et al., 2013; Lauer & Kandler, 1976; Palframan et al., 2003; Van Der Meulen et al., 2006). Pyruvate formed from D-glyceraldehyde-3-phosphate can be converted either into lactate by lactate dehydrogenase or into formate and acetyl-CoA by pyruvate formate lyase (Figure 6). Acetyl-CoA can serve as a precursor for ethanol or for acetate (Figure 6). While acetate formation generates additional ATP, the production of one mole of ethanol is linked to the regeneration of two NAD^+ (Figure 6). As more NAD^+ is regenerated via ethanol than via lactate formation, a metabolic shift of the bifid shunt towards ethanol is associated with higher carbon availability for acetate and thus ATP production (Palframan et al., 2003). The fermentation end product profile of *Bifidobacterium* strains has been suggested to depend on various parameters, including (i) the carbon source (Amaretti et al., 2007; De Vries & Stouthamer, 1968; González-Rodríguez et al., 2013; Liu et al., 2011; Palframan et al., 2003; Van Der Meulen et al., 2006), (ii) carbon availability (Lauer & Kandler, 1976), (iii) the specific consumption rate of the carbon source (Degnan & Macfarlane, 1994; Van Der Meulen et al., 2006), and (iv) the *Bifidobacterium* strain (**Paper I**) (De Vries & Stouthamer, 1968; Lauer & Kandler, 1976; Palframan et al., 2003; Van Der Meulen et al., 2006).

Whereas the pathway involving acetate, ethanol, formate, and lactate in bifidobacteria has already been characterized, the route leading to succinate formation remains poorly known (Van Der Meulen et al., 2006). Succinate is an intermediate of the tricarboxylic acid cycle. However, based on the absence of genes encoding 2-oxoglutarate dehydrogenase (EC 1.2.4.2), malate dehydrogenase (EC 1.1.1.37), and fumarase (EC 4.2.1.2), this cycle seems to be incomplete in bififobacteria (Figure 6) (**Paper I**) (Lee & O'Sullivan, 2010). According to *i*AZ480 and *i*MS520 predictions, succinate might be produced from fumarate via fumarate reductase (EC 1.3.5.4) or via fumarate-dependent dihydroorotic acid dehydrogenase (EC 1.3.98.1) during pyrimidine biosynthesis (**Paper I**). The required fumarate is predicted to be formed by adenylosuccinate lyase (EC 4.3.2.2.) in purine metabolism (**Paper I**).

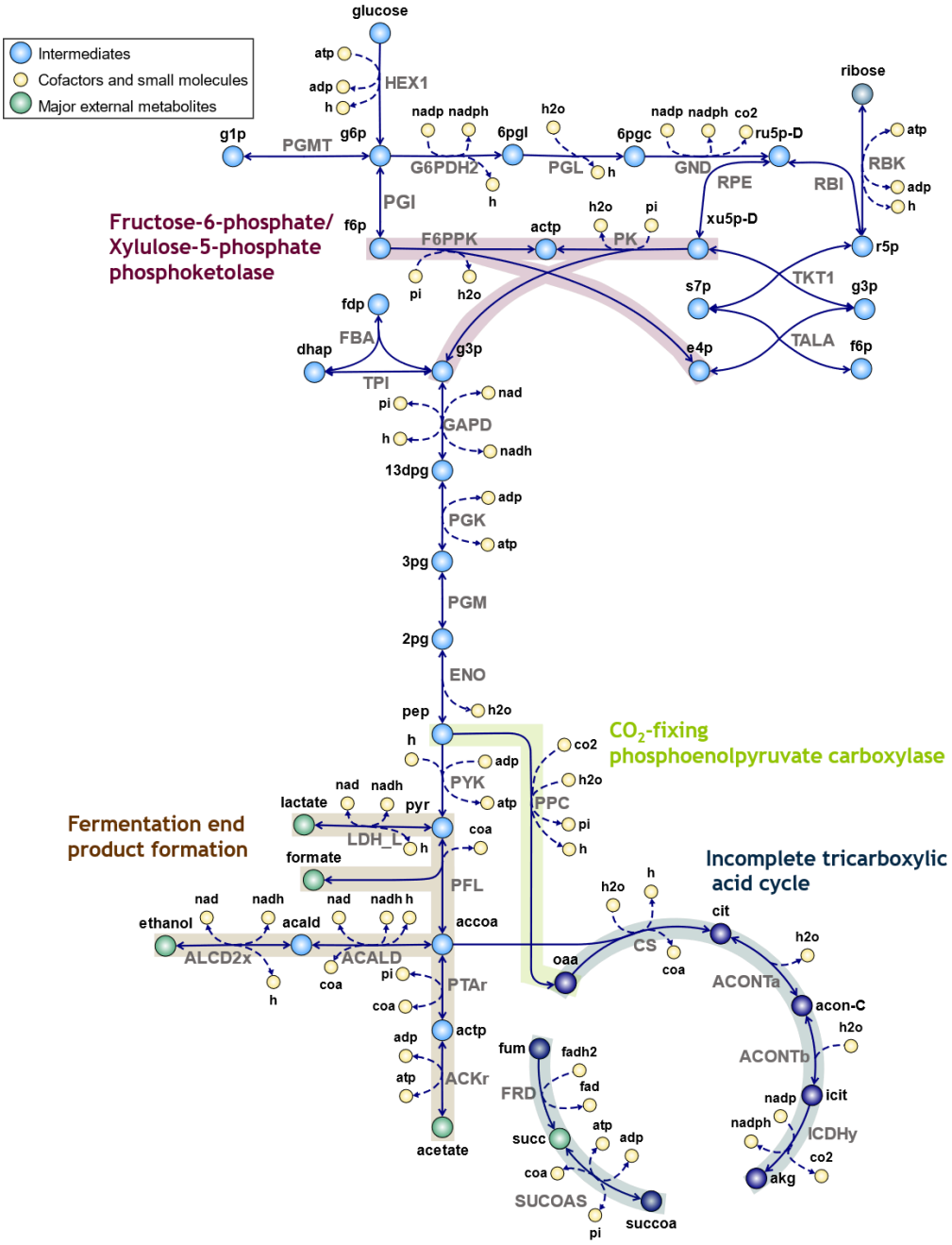


Figure 6: Central metabolism of bifidobacteria. The characteristic enzyme of the bifid shunt is xylulose-5-phosphate/fructose-6-phosphate phosphoketolase, which acts on xylulose-5-phosphate (xu5p-D) and fructose-6-phosphate (f6p) (PK, F6PPK). Key metabolites are abbreviated as follows: actp: acetyl-phosphate, e4p: erythrose-4-phosphate, g3p: glyceraldehyde-3-phosphate, pep: phosphoenol-pyruvate, oaa: oxaloacetate, pyr: pyruvate, accoa: acetyl-CoA, acald: acetaldehyde, cit: citrate: acon-C: cis-aconitate, icit: isocitrate, akg: 2-oxoglutarate, succoa: succinyl-CoA, succ: succinate, fum: fumarate. Key reactions are abbreviated as follows: PPC: phosphoenolpyruvate carboxylase, PYK: pyruvate kinase, PFL: pyruvate-formate lyase, PTAr: Phosphotransacetylase, ACKr: acetate kinase, ACALD: acetaldehyde dehydrogenase, ALCD2x: alcohol dehydrogenase, ACONTa/b; aconitase, ICDHy: isocitrate dehydrogenase, FRD; fumarase reductase, SUCOAS: succinyl-CoA synthetase. All other reactions and metabolites are abbreviated in accordance with the BIGG database (www.bigg.ucsd.edu) (adapted from Figure 1 in **Paper I** under the CC BY 4.0 license).

3.4 Nutritional requirements of bifidobacteria

Bifidobacteria are fastidious microorganisms in terms of their nutritional requirements (Fenster et al., 2019) and are auxotrophic for different types of nutrients, with requirements varying across species and strains.

Medium used for industrial-scale manufacturing often contains complex and undefined ingredients (Stanbury et al., 2013). However, knowing the nutritional requirement of a specific strain is essential to optimize the corresponding (Fenster et al., 2019). Previous formulations of chemically defined media (CDMs) for bifidobacteria did not support growth of BB-12 and BB-46 (Elli & Zink, 2002; Hassinen et al., 1951; Kongo et al., 2003; Sakaguchi et al., 2013). Therefore, *i*AZ480 and *i*MS520 were used to predict the nutritional requirements of BB-12 and BB-46 (**Paper I**). The predictions were validated and a CDM supporting growth of both strains was formulated (**Paper I**). The development of a CDM for probiotic bifidobacteria is associated with several advantages.

i. The CDM can be used for detailed physiological studies to quantify the effect of single ingredients on growth energetics and product yields, and to provide defined modeling conditions for the application of GEMs (**Paper I**). Such computational and physiological studies can help to optimize production processes, by identifying growth-promoting nutrients that can be added to the industrial medium to improve production efficiency.
ii. The CDM might serve as a pharma-grade alternative to currently used complex industrial media to produce strains with biotherapeutic applications (**Paper I**).
iii. The application of CDM might increase the reproducibility of the cultivation process, overcoming batch-to-batch variations in existing media. The higher costs typically associated with the use of a CDM in an industrial setting can be offset by a higher selling price of the produced strains, particularly for pharma-grade applications.

The current work highlighted that GENREs must be carefully curated to ensure reliable guidance for medium design by GEMs (**Paper I**).

The newly formulated CDM was used for most BB-12 and BB-46 cultivation experiments discussed in this thesis. Experimental data on the metabolism and physiology of the two strains were collected in anaerobic cultivations at 37°C performed either in crimp-top serum bottles (**Paper I**) or in lab-scale bioreactors (**Paper III, Paper IV**). Both types of cultivations were inoculated with exponentially growing cells precultured in crimp-top serum bottles. Cultivations in crimp-top serum bottles allowed a higher throughput. For cultivations in lab-scale bioreactors, the pH of the culture broth was maintained at pH 6.5, and the composition of the gas phase was kept at 20% carbon dioxide (CO_2) and 80% nitrogen (N_2). The same conditions were applied as starting conditions for crimp-top serum bottle cultivations. If not stated otherwise, sucrose was used as the main carbon and energy source because it supported a consistently high biomass concentration (1.7 $g_{CDW}\ L^{-1}$) for both strains cultivated in crimp-top serum bottle (**Paper I**).

In the following paragraphs the nutritional requirements of bifidobacteria are describe in greater detail, especially of BB-12 and BB-46, in terms of carbon source, vitamins, amino acids, and CO_2.

Carbon source. Bifidobacteria can catabolize a variety of mono- and oligosaccharides as carbon and energy source, in a manner that varies among species and strains (de Vrese & Schrezenmeir, 2008; Mattarelli & Biavati, 2018; Pokusaeva et al., 2011). The capability of bifidobacteria to utilize oligosaccharides such as fructooligosaccharides (Rossi et al., 2005) as carbon and energy source reflects their adaptation to non-digestible carbohydrates in the gastrointestinal tract of their host (Schell et al., 2002). Such complex carbohydrates can be broken down into monosaccharides by a variety of carbohydrate-active genes, including glycosyl hydrolases and hexosyltransferases, encoded in the genome of bifidobacteria (Pokusaeva et al., 2011). Due to the growth-promoting effect of some non-digestible oligosaccharides, such as fructooligosaccharides, and galactooligosaccharides, they are considered bifidogenic prebiotics and can be administered to selectively promote the growth of bifidobacteria in the gastrointestinal tract (de Vrese & Schrezenmeir, 2008).

In **Paper I**, the capability of BB-12 and BB-46 to utilize 12 different mono- and oligosaccharides from CDM was tested in crimp-top serum bottles. BB-46 grew on all substrates except mannose; whereas BB-12 did not catabolize arabinose, fructose, mannose, and xylose. Neither strain could use amino acids as sole carbon and energy source (**Paper I**).

Vitamins. Bifidobacteria synthesize water-soluble vitamins, such as folic acid and nicotinic acid, and secrete them into the medium, although such ability varies across strains and species (D'Aimmo et al., 2012; Deguchi et al., 1985; Pompei et al., 2007; Sugahara et al., 2015). Consequently, the requirement for vitamins in the growth medium can be strain dependent, and was found to differ between BB-12 and BB-46 (**Paper I**).

Specifically, BB-46 grew in CDM lacking nicotinic acid or nicotinamide; whereas BB-12 showed an absolute requirement for nicotinic acid and an inability to utilize nicotinamide as a precursor (**Paper I**) owing to absence of a gene coding for nicotinamidase (EC 3.5.1.19). Based on the KEGG database, the feature seems common to *B. animalis* (**Paper I**).

The requirement for menaquinone-4 (vitamin K_2) was also found to differ between BB-12 and BB-46, both of which lack genes for vitamin K biosynthesis (**Paper I**). While BB-46 needed menaquinone-4 for growth, BB-12 grew also in its absence. However, the lag phase and final biomass yield of BB-12 could not be reproduced when cultivated over several passages in CDM without menaquinone-4 (**Paper I**). Different forms of vitamin K including menaquinone-4 as well as vitamin K-related compounds such as 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) promote growth of *Bifidobacterium* strains (Glick et al., 1959; Hojo et al., 2007; Kaneko, 1999). The latter, in particular, has been suggested to act as an electron transfer mediator for $NAD(P)^+$ regeneration and detoxification of hydrogen peroxide (H_2O_2) (Kaneko, 1999; Yamazaki et al., 1999). The GEMs of BB-12 and BB-46 suggested that vitamin K might serve as cofactor in some redox reactions such as the one catalyzed by dihydroorotate dehydrogenase (EC 1.3.5.2) (**Paper I**). In addition, the genome of BB-12 and BB-46 harbors a gene annotated as NADPH-quinone oxidoreductase (EC 1.6.5.5) that uses quinone as a redox mediator to oxidize NADPH to $NADP^+$ (**Paper I**).

However, the function of menaquinone-4 in the bifidobacterial metabolism and its requirement by BB-46 remain to be determined.

In contrast to the previous two examples, BB-12 and BB-46 shared some other vitamin requirements. Unlike other *Bifidobacterium* strains (Hassinen et al., 1951; Yoshioka et al., 1968), BB-12 and BB-46 are unable to use pantothenate and need pantethine, the stable disulfide dimer of pantothenate, as precursor for coenzyme A biosynthesis (**Paper I**). The inability to use pantothenate was explained by the absence of genes encoding phosphopantothenate-cysteine ligase (EC 6.3.2.5) and phosphopantothenyl cysteine decarboxylase (EC 4.1.1.36), which catalyze the conversion of phosphopantothenate into pantethine-4-phosphate (Figure 7). Based on genome sequences available from the KEGG database, the inability to use pantothenate by bifidobacteria seemed to be widely species-dependent (**Paper I**).

The examples of nicotinic acid requirement (for BB-12) and pantethine (for BB-12 and BB-46) emphasize how the form of the supplied vitamin is crucial for growth of bifidobacteria.

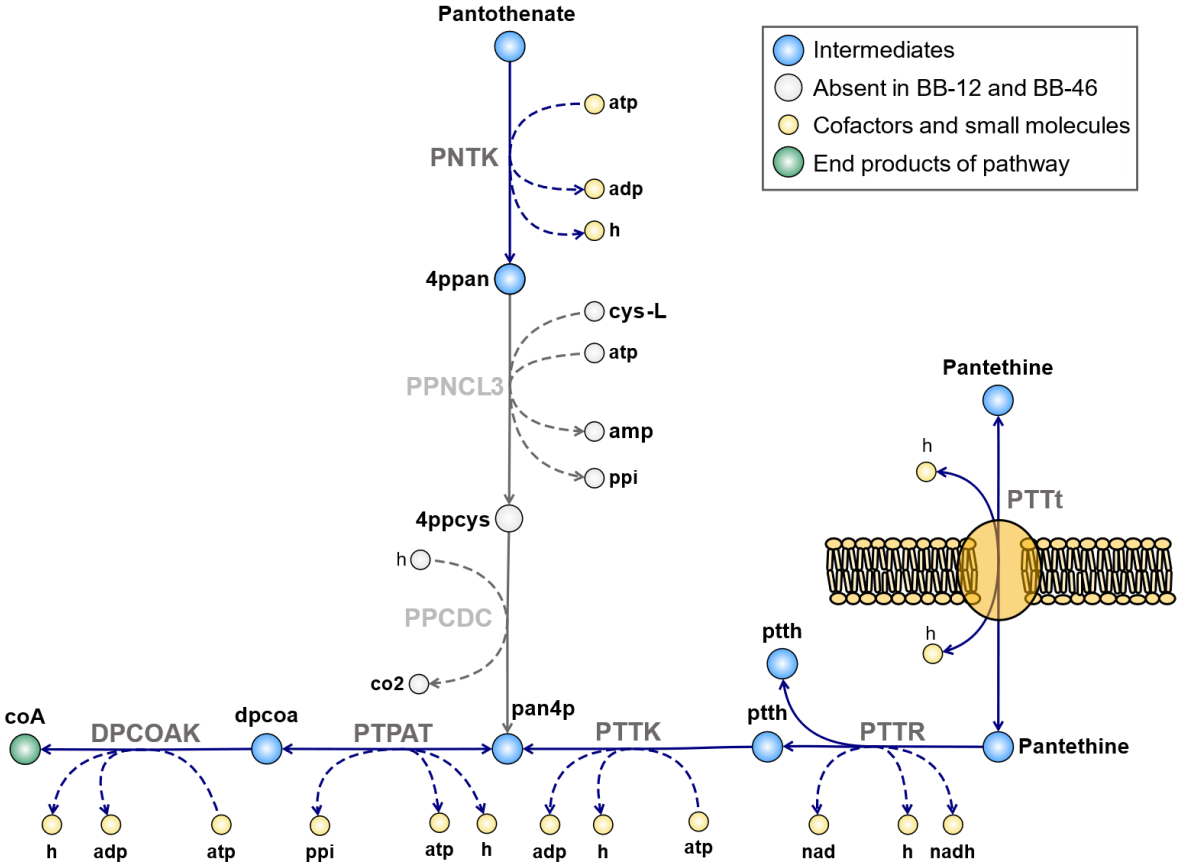


Figure 7: Biosynthesis of coenzyme A in bifidobacteria. Proposed pathway of coenzyme A (coA) biosynthesis from pantethine in BB-12 and BB-46. The two strains cannot use pantothenate as precursor for coenzyme A biosynthesis due to the absence of phosphopantothenate-cysteine ligase and phosphopantothenyl cysteine decarboxylase, which catalyze the conversion of phosphopantothenate (4ppan) into pantethine-4-phosphate (pan4p) through the PPNCL3 and PPCDC-mediated reactions. Abbreviations of reactions and metabolites are in accordance with the BIGG database (adapted from Figure 4 in **Paper I** under the CC BY 4.0 license).

Amino acids. Bifidobacteria have been described to require L-cysteine as an organic sulfur source (Ueda et al., 1983) and to lack the ability for the assimilation of inorganic sulfur, due to the absence of key genes involved in the assimilation of sulfate and sulfide, such as ATP sulfurylase (EC. 2.7.7.4) (Ferrario et al., 2015; Hassinen et al., 1951; Lee & O'Sullivan, 2010; Schell et al., 2002). At the same time, none of the other proteinogenic amino acids seem to be required for growth of most *Bifidobacterium* strains (Ferrario et al., 2015; Hassinen et al., 1951; Ueda et al., 1983). One exception is *Bifidobacterium longum* subsp. *longum* 105-A, which needs also tyrosine and isoleucine (Sakaguchi et al., 2013).

In agreement with most *Bifidobacterium* strains, BB-12 and BB-46 were predicted and validated to grow in CDM with L-cysteine as the sole sulfur source. However, in contrast to previous study (Hassinen et al., 1951), L-methionine could replace L-cysteine as sole amino acid and sulfur source (**Paper I**), echoing another recent study showing that several *Bifidobacterium* strains can grow in medium with L-methionine as sole sulfur source (Wada et al., 2021). Based on the GEMs of BB-12 and BB-46, putative routes for L-cysteine biosynthesis from L-methionine, and vice versa, were proposed (Figure 8) (**Paper I**).

In BB-12, which lacks a gene encoding adenosylhomocysteinase (EC 3.3.1.1), the synthesis of L-cysteine from L-methionine is strictly linked to the activity of LuxS (EC 4.4.1.21), which catalyzes the conversion of S-ribosylhomocysteine (rhcys) to homocysteine (hcys-L)

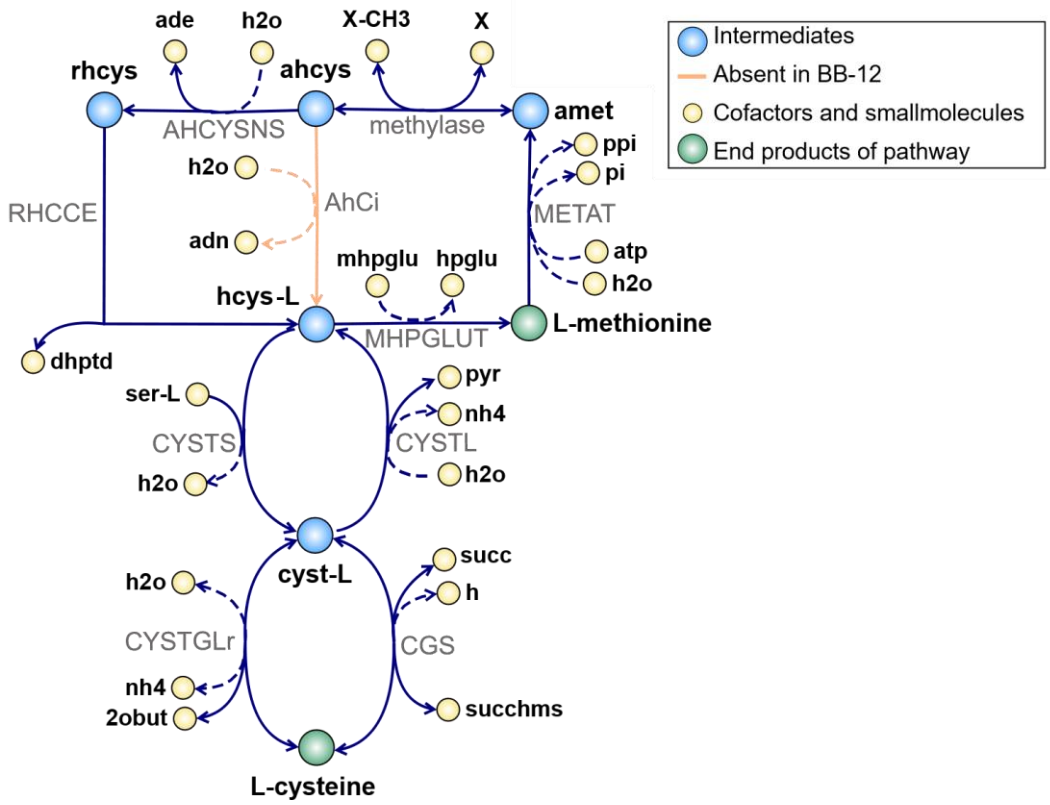


Figure 8: Interconversion of L-cysteine and L-methionine. Proposed pathway of L-cysteine biosynthesis from L-methionine, and vice versa, in BB-12 and BB-46. Abbreviations of reactions and metabolites are in accordance with the BIGG database (adapted from Figure 6 in **Paper I** under the CC BY 4.0 license).

and 4,5-dihydroxy-2,3-pentadione (dhptd) (RHCEE) (Figure 8). The latter is a precursor of autoinducer II and LuxS activity has been shown to enhance biofilm formation in *B. longum* NCC2795 (Sun et al., 2014). Accordingly, a limited supply of L-cysteine in the presence of L-methionine might induce biofilm formation in some *Bifidobacterium* strains.

When added to growth medium, L-cysteine serves as a reducing agent, amino acid, and sulfur-source. In the absence of L-cysteine, L-ascorbic acid was found to function as a suitable alternative reducing agent (**Paper I**). Even though only one of the sulfur-containing amino acids appears to be essential for growth of bifidobacteria, supply of additional amino acids had a growth-promoting effect on BB-12 and BB-46 (**Paper I**).

Instead of sulfate and sulfite, bifidobacteria might be able to use hydrogen sulfide (H_2S) as a sulfur source through the action of an O-succinyl-L-homoserine succinate-lyase (EC 2.5.1.48) (Lee & O'Sullivan, 2010; Schell et al., 2002). Whereas this hypothesis was predicted by *i*AZ480 and *i*MS520 (**Paper I**), neither BB-12 nor BB-46 were capable of using H_2S as the sole sulfur source when fed a H_2S-releasing compound (GYY4137) (**Paper I**).

Besides single amino acids, peptides, such as those obtained from hydrolyzed casein, can also be used as nitrogen source by bifidobacteria (Hassinen et al., 1951; Kongo et al., 2003). Indeed, *i*AZ480 and *i*MS520 predicted the ability of BB-12 and BB-46 to use peptides as a source of free amino acids (**Paper I**). In **Paper IV**, the replacement of all free amino acids except L-cysteine with casein hydrolysate was found to promote growth of BB-12 and BB-46 (section 4.4), confirming earlier findings in other *Bifidobacterium* strains (Zhang et al., 2020).

Carbon dioxide. Based on evidence showing a requirement (in solid medium) or growth-promoting effect of CO_2 on *Bifidobacterium* strains (Biavati & Mattarelli, 2015; Kawasaki et al., 2007; Tamura, 1983), cultivations commonly include CO_2-containing gas mixtures (De Vries & Stouthamer, 1967; Ueda et al., 1983; Van Der Meulen et al., 2006). Whereas the molecular mechanism underlying the need of some *Bifidobacterium* strains for CO_2 remains unclear, the use of CO_2 by *B. longum* JBL05 was associated solely with growth and production of exopolysaccharides in the absence of oxaloacetate (Ninomiya et al., 2009). This finding suggests that CO_2 is needed for the activity of phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31), which catalyzes the carboxylation of PEP to oxaloacetate (Figure 6) (Ninomiya et al., 2009).

The ability of BB-12 and BB-46 to grow in the absence of CO_2 was tested in crimp-top serum bottles in the newly formulated CDM without carbonate (unpublished results). Under this condition, BB-12 and BB-46 showed similar growth behavior as in the presence of CO_2 and carbonate. However, when BB-12 and BB-46 were cultivated in the same medium in lab-scale bioreactors, none of the strains grew. The discrepancy might be explained by differences in the control of gas composition. While the headspace of the bioreactors was sparged with N_2 throughout the cultivation, the headspace of the crimp-top serum bottles was only flushed during their preparation to attain anaerobic conditions. When cultivated in serum bottles (closed system), the two strains might produce sufficient CO_2 to fuel essential CO_2-fixing reactions, whereas CO_2 produced in bioreactors is removed due to continuous flushing of the head space. The GEMs of BB-12 and BB-46 predicted that CO_2 was formed via fatty acid biosynthesis and by 6-phosphogluconate dehydrogenase (EC 1.1.1.44), and

that in the absence of sufficient purines, CO_2 is required by phosphoribosyl-aminoimidazole carboxylase (EC. 4.1.1.21) for purine biosynthesis.

Based on above evidence, in this work, the ability of BB-12 to grow in lab-scale bioreactors without CO_2 and carbonate, and at continuous N_2-sparging, was assessed following the addition of 0.75 g L^{-1} oxaloacetate to the medium (unpublished data). In this case, BB-12 exhibited a reduced specific growth rate (μ_{max} = 0.15 h^{-1}) and large variations in the final biomass yield. This result indicates that, as in *B. longum* JBL05, BB-12 can bypass the requirement for CO_2 through addition of oxaloacetate. Based on the GEM prediction, the supply of purines in the medium may improve growth of the strain in the absence of CO_2; however, this hypothesis awaits experimental validation.

Knowledge of the nutritional requirements of BB-12 and BB-46 obtained in this thesis is summarized in Table 1.

Table 1. Differences and similarities between BB-12 and BB-46 with respect to their nutritional requirements (**Paper I**)**.**

	BB-12	**BB-46**
Carbohydrates supporting growth	Sucrose, maltose, raffinose, melibiose, lactose, glucose, galactose, ribose	Sucrose, maltose, raffinose, melibiose, lactose, glucose, fructose, galactose, ribose, arabinose, xylose
Carbohydrates not supporting growth	Fructose, mannose, arabinose, xylose	Mannose
Essential vitamins	Pantethine, nicotinic acid, folate	Pantethine, menaquinone-4, PABA(1) or folate
Non-essential vitamins	Pyridoxal, lipoic acid, menaquinone-4	Pyridoxal, lipoic acid, nicotinamide, nicotinic acid
Vitamins not supporting growth	Nicotinamide, PABA(1), pantothenate	Pantothenate
Amino acids	Requirement for either L-cysteine or L-methionine	Requirement for either L-cysteine or L-methionine
CO_2 requirement	No requirement in the closed system of a crimp-top serum bottle; required in a continuously flushed bioreactor in the absence of oxaloacetate	No requirement in the closed system of a crimp-top serum bottle; required in a continuously flushed bioreactor. Effect of oxaloacetate has not been tested
Additional non-essential nutrients(2)	Polyamines (spermidine, putrescine), nucleobases (uracil, guanine, adenine, xanthine, hypoxanthine, and inosine), orotic acid, L-ornithine, and N-acetyl-glucosamine	Polyamines (spermidine, putrescine), nucleobases (uracil, guanine, adenine, xanthine, hypoxanthine, and inosine), orotic acid, L-ornithine, and N-acetyl-glucosamine

(1) PABA: *para*-aminobenzoic acid, (2) Nutrients suggested to be essential based on literature or previous versions of *i*AZ480 and *i*MS520.

3.5 Growth characteristics of BB-12 and BB-46

A detailed understanding of the growth characteristics of probiotic bifidobacteria will favor their industrial application. In this work, the growth characteristics of BB-12 and BB-46 were studied first in CDM using crimp-top serum bottles (**Paper I)**. Growth dynamics and metabolite production varied among the two strains when cultivated under these conditions (Figure 9, Table 2). BB-12 reached a maximum specific growth rate of 0.45 h^{-1} and BB-46 of 0.35 h^{-1} (Table 2). Moreover, while acetate and lactate were the main metabolites produced by BB-12 throughout the entire cultivation; BB-46 secreted mainly acetate, formate, and ethanol in the exponential phase (Figure 9) In the deceleration phase, BB-46 started producing lactate, while formate and ethanol production ceased (Figure 9). These results echo previous studies, whereby different *Bifidobacterium* strains exhibited different metabolite profiles under the same cultivation conditions (De Vries & Stouthamer, 1968; Palframan et al., 2003; Van Der Meulen et al., 2006). A higher specific uptake rate of the carbon source has been linked to an increased production of lactate, but reduced production of acetate, formate, and ethanol (Van Der Meulen et al., 2006). Indeed, BB-12, which showed 36% higher sucrose uptake than BB-46, displayed higher lactate, formate, and ethanol secretion in the exponential phase (Table 2). Nevertheless, the specific acetate production rates were similar for BB-12 and BB-46 (Table 2). During cultivation, the pH of the medium dropped to 4.02 ± 0.1 (BB-12) and 4.04 ± 0.3 (BB-46). While BB-12 consumed all the supplied sucrose (20 mM), traces of sucrose were still detected in the medium of BB-46 in stationary phase (Figure 9), which might be explained by higher acid sensitivity of BB-46 compared to BB-12.

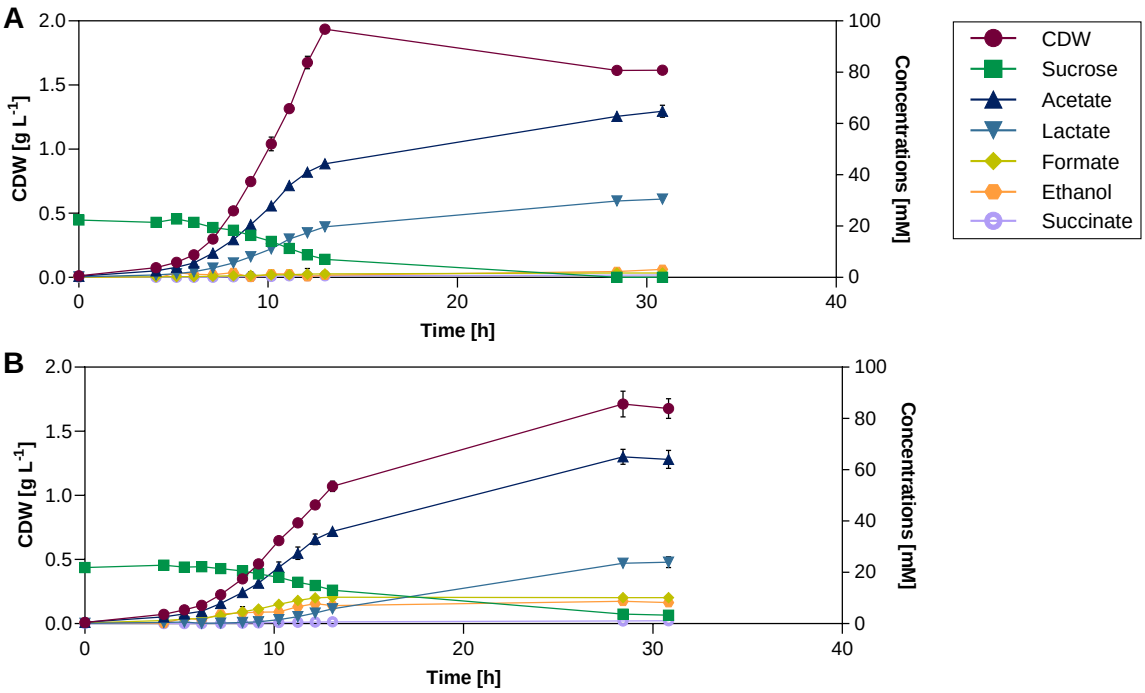


Figure 9: Growth dynamics and metabolite profiles of BB-12 (A) and BB-46 (B). Cultivations were conducted in crimp-top serum bottles under anaerobic conditions (80% N_2 and 20% CO_2) at 37°C in CDM supplemented with 10 g L^{-1} sucrose and a starting pH of 6.5. Each data point represents the mean of biological triplicates ± standard deviation.

Table 2: Comparison of *in vitro* and *in silico* reaction rates predicted by *i*AZ480 and *i*MS520 for cultivation of BB-12 and BB-46 in the newly formulated CDM (adapted from Table 2 in **Paper I** under the CC BY 4.0 license).

	BB-12 ***in vitro***	**BB-12** ***in silico***	**BB-46** ***in vitro***	**BB-46** ***in silico***
Specific growth rate [h^{-1}]	0.45 ± 0.0	0.44	0.35 ± 0.0	0.34
Sucrose uptake rate[1)] [mmol g_{CDW}^{-1} h^{-1}]	3.9 ± 0.2	3.9 [1)]	2.8 ± 0.4	2.8 [1)]
Acetate:lactate ratio[1)] [-]	2.5 ± 0.0	2.5 [1)]	17.3 ± 0.0	17.3 [1)]
Acetate secretion rate [mmol g_{CDW}^{-1} h^{-1}]	11.8 ± 0.7	12.0	11.9 ± 0.3	10.4
Lactate secretion rate [mmol g_{CDW}^{-1} h^{-1}]	4.7 ± 0.3	4.8	0.7 ± 0.0	0.6
Formate secretion rate [mmol g_{CDW}^{-1} h^{-1}]	0.3 ± 0.0	3.3	4.0 ± 0.2	5.8
Succinate secretion rate [mmol g_{CDW}^{-1} h^{-1}]	0.2 ± 0.0	0.2	0.2 ± 0.0	0.1
Ethanol secretion rate [mmol g_{CDW}^{-1} h^{-1}]	-	0.4	2.5 ± 0.3	1.7
Carbon recovery excluding biomass formation [%]	82	-	106	-

[1)] The sucrose uptake rate and the acetate:lactate ratio were constrained to the experimentally determined values. Amino acid uptake rates were constrained to 1 mmol g_{CDW}^{-1} h^{-1}. The carbon recovery was calculated based on specific rates determined during the exponential growth phase and does not include biomass formation. *In vitro* reaction rates are given as means ± standard deviations of three replicates.

The measured flux rates of exponentially growing BB-12 and BB-46 in CDM (Table 2) were used to calculate the growth- and non-growth-associated maintenance energy requirements in the BOF of *i*AZ480 and *i*MS520, and to quantitatively validate the predictions of the models (**Paper I**). Very good agreement was found between *in vitro* and *in silico* reaction rates when BB-12 and BB-46 were cultivated in CDM; only the formate production rate was higher *in silico* than *in vivo* (Table 2) (**Paper I**). The reason for the underestimated formate production or its conversion to another compound needs to be examined further.

When cultivated under pH-controlled conditions in lab-scale fermenters, BB-12 showed a similar maximum specific growth rate (0.42 ± 0.02 h^{-1}) and predominance of acetate and lactate (with an acetate:lactate ratio of 1.95 ± 0.03) among secreted products, as under non-pH-controlled conditions (Table 2) (**Paper III**). In contrast to non-pH-controlled conditions, BB-46 displayed no metabolic shift towards lactate during the deceleration phase and secreted acetate and formate as main fermentation end products throughout the entire cultivation under pH-controlled conditions (**Paper III**). The metabolic shift from formate towards lactate under non-pH-controlled conditions (Figure 9) might be explained by the different acidity of the two compounds. Because formate (pKa of 4.3) is a weaker acid than lactate (pKa of 3.8), a lower pH implies a higher molar fraction of undissociated formate than lactate. As undissociated weak acids can passively cross the cell membrane, they can promote the acidification of the cytoplasm. Hence, blocking formate secretion at decreasing pH might lower the acidic stress for the target strain. However, this hypothesis remains to be validated, and gives rise to the question of how the cells might sense and signal the need for a change in metabolic flux.

Paper IV highlights how the metabolite profile of BB-12 was not considerably affected by a change in medium composition; whereas that of BB-46 responded to the presence of a complex nitrogen source, as discussed in detail in section 4.4.

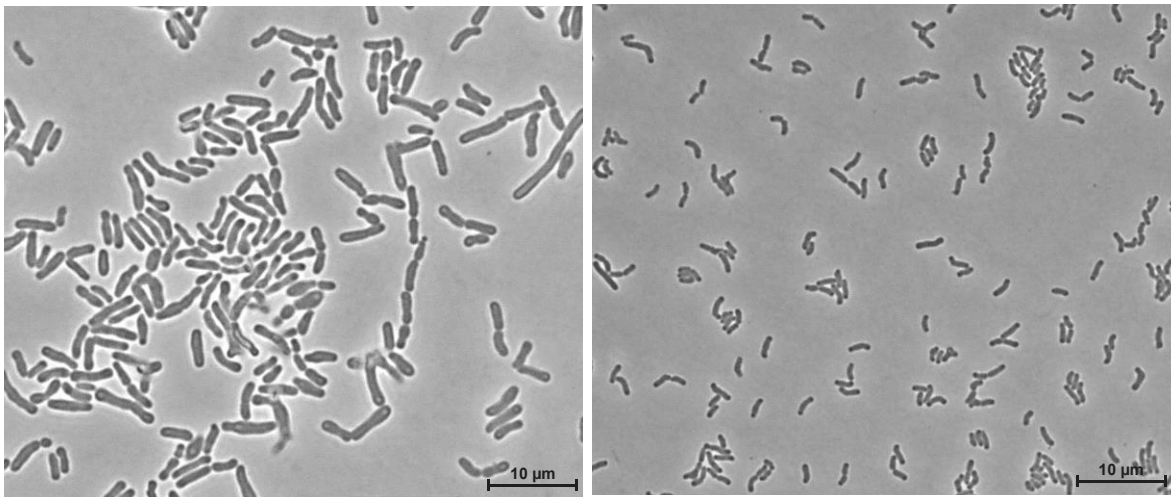


Figure 10: Microscopy image of BB-12 (left) and BB-46 (right) taken during early exponential growth in CDM. The image was taken with a 100× 1.4 NA phase-contrast objective by Dr. Bech-Terkilsen.

3.6 Morphology of bifidobacteria

Bifidobacteria are pleomorphic and can form straight, multi-branched or Y-shaped (bifid) rods (Bezkorovainy & Miller-Catchpole, 1989). Their morphology is not only species-dependent but can vary also with respect to growth conditions (Bezkorovainy & Miller-Catchpole, 1989; Dhanashree et al., 2017).

The morphology of BB-12 and BB-46 during the early exponential growth in CDM is shown in Figure 10. BB-12 cells were markedly larger and presented greater variation in length than BB-46 cells (Figure 10). Both strains formed straight rods and no branching was observed. Branched bifidobacteria have been linked to lack of essential precursors for cell wall synthesis in the growth medium (Bezkorovainy & Miller-Catchpole, 1989). Therefore, the absence of branched BB-12 and BB-46 in CDM may prove a complete cell wall synthesis under the given conditions.

3.7 Summary

Taken together, high-quality GEMs of BB-12 and BB-46 were reconstructed to describe the metabolic capabilities of the two strains (**Paper I**). The manually curated GEMs were used to identify the nutritional requirements for carbon sources, vitamins, and amino acids of BB-12 and BB-46. This knowledge was applied to formulate a CDM that supported growth of both strains (**Paper I**). When cultivated in the newly developed medium, BB-12 and BB-46 showed different metabolite profiles (**Paper I**). The GEMs and the newly developed medium represent valuable tools to study the metabolism and physiology of *Bifidobacterium* strains.

4 Variability in stress physiology of bifidobacteria

During their life span from cultivation to administration, bifidobacteria encounter a variety of stressors, such as O_2, which can decrease viability (section 2.4). The tolerance of *Bifidobacterium* strains towards such stressors has been shown to differ considerably between species but also between strains of the same species (Mättö et al., 2004; Simpson et al., 2005). The production of a probiotic *Bifidobacterium* strain that shows rather poor robustness and stability is highly challenging as the strain's sensitivities need first to be understood before the manufacturing process can be tailored to its requirements. In the last decades, several studies have investigated the response of bifidobacteria to stressors. Further improvements to our understanding of the stress physiology of clinically and industrially relevant bifidobacteria will facilitate the design of more robust and stable strains (see Box. 1 in section 1.1).

4.1 Assessment of robustness and stability

An essential element of studies on the stability and robustness of microorganisms is the accurate quantification of cell survival during exposure to stressors and storage. Traditionally, viability is equated with culturability (Kell et al., 1998; Pinto et al., 2015). Therefore, conventional plating techniques are commonly applied as a growth-dependent method to investigate the viability of probiotics (Bertazzoni et al., 2013; Egan et al., 2018; Hill et al., 2014; Mozzetti et al., 2013; Xiao et al., 2011).

Recent challenges to the conventional definition of viability have led scientists to uncouple culturability from viability (Pinto et al., 2015). The new definition of viability focuses mainly on the 'viable but non-culturable' physiological state, in which microorganisms retain their (metabolic) activity and cell integrity but cannot replicate until they have been subjected to a resuscitation process (Kell et al., 1998). However, it has been suggested to replace the term 'viable but non-culturable' with alternative expressions to maintain the direct connection between the terms culturability and viability (Kell et al., 1998). It has been proposed that a cell could be present in the physiological states summarized in Table 3.

Table 3: Physiological state of microorganisms according to Kell and coworkers (Kell et al., 1998).

Physiological state	Definition
Viable	State in which cells are metabolically active and are immediately culturable.
Dormant	Reversible state in which the cells are (almost) metabolically inactive. Cells are (ultimately) culturable.
Active but non-culturable (ABNC)	State in which the cells are metabolically active but do not grow to a detectable level. Recovery of a culturable state has not been demonstrated. Cells cannot regain culturability.
Not immediately culturable (NIC)	State in which the cells are metabolically active but do not grow to a detectable level. Cells have been shown to be ultimately culturable (retrospective detection of the NIC state).
Sub-lethally injured	Damaged cells that require reparative processes to (re)grow.

Even though a few studies have proposed that probiotic *Bifidobacterium* strains can enter a state, whereby they retain metabolic activity and membrane integrity but are non-culturable (Hansen et al., 2018; Lahtinen et al., 2005, 2006), there is no evidence indicating that bifidobacteria can regain their culturability (resuscitation) after having entered this state, rather than just dying. Thus, cells that have entered this state might eventually die. Moreover, to increase the comparability of scientific studies on the robustness and stability of bifidobacteria, standardized methods for the assessment of viability are essential. Therefore, a traditional growth-dependent plating technique was chosen in this project to assess the viability of BB-12 and BB-46 (**Paper III, Paper IV**). However, it was coupled with flow cytometry to assess membrane integrity and to gain valuable information on the physiological state of BB-12 and BB-46 cultures (**Paper III**).

To compare the robustness and stability of BB-12 and BB-46 in this work, a stress test was established (Figure 11). The test was set up to meet the following requirements:

i. capture the physiological state of the strains directly after cultivation,
ii. be unaffected by bottlenecks in the capacity of devices used for downstream processing,
iii. demonstrate differences in robustness and stability between strains, and
iv. be relatively quick.

To fulfill these requirements, the survival of BB-12 and BB-46 was assessed during short-term storage in solution. The cells were kept either in culture broth (**Paper III**) or in a peptone-saline solution (pH 7.0) and protected from light (Figure 11) (**Paper IV**). The number of viable cells before and after storage was determined by CFU counts. The loss of CFUs during storage was calculated to determine cell survival (**Paper III, Paper IV**).

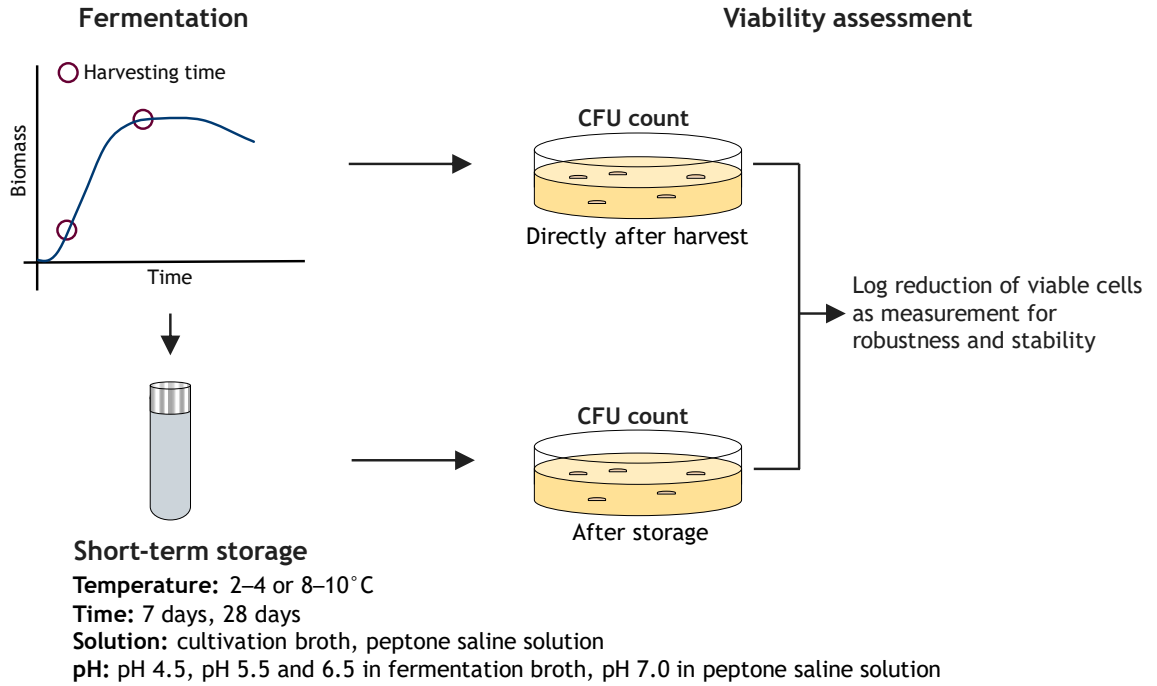


Figure 11: Stress test used for the assessment of robustness and stability of BB-12 and BB-46. CFU: colony forming units.

Storage conditions were chosen to investigate strain tolerance towards several stressors. The cells were kept at low temperatures (2–4°C or 8–10°C) under aerobic conditions (oxidative stress) (Figure 11). A pour plating method was used for CFU determination, meaning that the strains were exposed to heat shock when in contact with 46 ± 1°C molten agar medium. While keeping these parameters constant, the effect of storage pH (pH 4.5–6.5), storage time (7–28 days), and the harvesting time point (exponential or stationary phase) was investigated (Figure 11) (**Paper III**), together with the effect of starvation and acid stress. Under the chosen storage conditions, the strains were expected to be in a vegetative state, like the one they enter when included in wet food products such as yoghurt (Lee et al., 2009).

The log reduction in viable cells during storage was used as a combined measurement of robustness and stability (Figure 11). Robustness, which denotes the cells' ability to sustain their functions despite being exposed to perturbations (**Paper II**), is the consequence of tolerance towards individual stressors. In the applied stress test, robustness determined the strains' survival directly after harvesting, when the cells were subjected to stressors such as O_2. Stability, which denotes the cells' ability to remain viable under given environmental conditions encountered during storage (**Paper II**), was assessed by maintaining the cells for up to 28 days at these conditions. As strain survival was tested only at the end of storage and not also after transferring the cells from the bioreactor to the storage condition, no independent measurement of their robustness and stability was obtained.

Other stress tests have been applied in previous studies to test the robustness/stress tolerance and stability of bifidobacteria. Similar to this work, Lahtinen and coworkers have applied a stress test based on survival of non-preserved cells during short-term storage (Lahtinen et al., 2006). In other studies, the stability of strains during storage has been tested by comparing the CFU count of freeze-dried cells before and after different storage times in different media (Saarela et al., 2005), such as juices, milk or phosphate-buffered saline (Saarela et al., 2006). Stress tolerance of *Bifidobacterium* strains has been commonly assessed by following the decay in viability determined by CFU counts upon exposure to a stressor (Mättö et al., 2004; Oberg et al., 2011; Simpson et al., 2005; Vernazza et al., 2006) or by testing the ability of a strain to grow in the presence of a stressor (Kawasaki et al., 2006; Simpson et al., 2005; Tanaka et al., 2018).

4.2 Variation in stress tolerance of bifidobacteria

Bifidobacteria demonstrate differences not only with respect to nutritional requirements (section 3.4), but also with regard to their tolerance to stressors, which determines their overall robustness. For example, O_2 sensitivity of bifidobacteria ranges from O_2-hypersensitivity, whereby growth is inhibited by O_2 concentrations below 5.0%, to O_2-hypertolerance, whereby species can grow in the presence of 20.0% O_2 (Kawasaki et al., 2018). *B. longum* subsp. *longum* strains are generally classified as O_2-sensitive, as they cannot grow in presence of 5.0% O_2, whereas *Bifidobacterium animalis* subsp. *lactis* strains are considered O_2-tolerant, as they grow in the presence of 10.0%–17.5% O_2 (Kawasaki et al., 2018). Acid tolerance can also vary greatly among *Bifidobacterium* strains (Matsumoto et al., 2004; Mättö et al., 2004; Vernazza et al., 2006). BB-12 has been found to survive at pH 2 for 20 min; whereas low or even no survival has been observed for strains of the species *B. longum* and *B. adolescentis*, when exposed to pH 3 or pH 4 for 10 min (Vernazza et al.,

2006). Variations in stress tolerance have been detected even between strains of the same species (Mättö et al., 2004; Simpson et al., 2005).

Probiotic cultures are commonly harvested in the deceleration or stationary phase to maximize the final cell density (Saarela et al., 2004). Besides dictating the biomass yield, the harvesting time can affect also the stress tolerance of a strain. While survival of *B. longum* R0175 during freezing has been shown to be higher for cells harvested in stationary as opposed to exponential phase (Louesdon et al., 2015), no such effect has been reported for freeze-drying tolerance of BB-12 (=E-012010) (Saarela et al., 2005).

The present work explored the stress physiology of BB-12 and BB-46. As highlighted by the examples given above, BB-12 is equipped with exceptionally high robustness and stability (Jungersen et al., 2014; Simpson et al., 2005; Vernazza et al., 2006). In contrast, *B. longum* strains show high sensitivity towards stressors, such as O_2 and acids (Kiviharju et al., 2004; Simpson et al., 2005; Vernazza et al., 2006).

The superior robustness and stability of BB-12 compared to BB-46 was confirmed when applying the stress test described in section 4.1 to cells cultivated in the newly formulated CDM (**Paper III**). As summarized hereafter, the harvesting time point, storage time and storage pH were shown to affect, to a varying extent, the survival of BB-12 and BB-46 (Figure 12).

i. In line with previous findings, the harvesting time affected only the survival of BB-46 but not that of BB-12 when stored for 7 days at pH 6.5 and at 8–10°C. BB-46 cells harvested in exponential phase exhibited 90% viability loss (1.0 ± 0.4 $\log_{10}$ loss), whereas those harvested in stationary phase showed no viability loss (Figure 12A). Hence, growth phase-dependent stress tolerance appears to be a shared characteristic of *B. longum* strains, although this remains to be validated.
ii. After extended storage for 28 days at pH 6.5 and at 8–10°C, BB-12 displayed only 80% viability loss (0.7 ± 0.2 $\log_{10}$ loss), whereas no viable BB-46 cells were detected under the same conditions (Figure 12B).
iii. For both strains, survival decreased with decreasing storage pH, from pH 6.5 to pH 4.5 (Figure 12C). While survival of the two strains was comparable at pH 6.5 and pH 5.5, BB-12 showed considerably better survival than BB-46 at pH 4.5 (Figure 12C).
iv. Storage temperature affected the survival of BB-46 during short-term storage (**Paper IV**). While no viable cells were detected after storage at 10°C for 28 days under aerobic conditions in peptone saline solution, survival appeared better (4.3 ± 1.7 $\log_{10}$ loss) following storage at 4°C.

Besides CFU counts, the number of cells with intact membrane (active cells) was determined by flow cytometry (**Paper III**). The number of active BB-12 cells was in good agreement with the CFU count determined after storage across the tested conditions (**Paper III**). In contrast, the number of active BB-46 cells exceeded the CFU count in the samples stored for 28 days at pH 6.5 and for 7 days at pH 4.5 (**Paper III**). These results suggest that BB-46 cells entered a ‘not immediately culturable’, ‘dormant’ or ‘active but non-culturable’ state under these conditions (Table 3) (**Paper III**). However, as mentioned in section 4.1, the existence and implication of these states in bifidobacteria remain unclear.

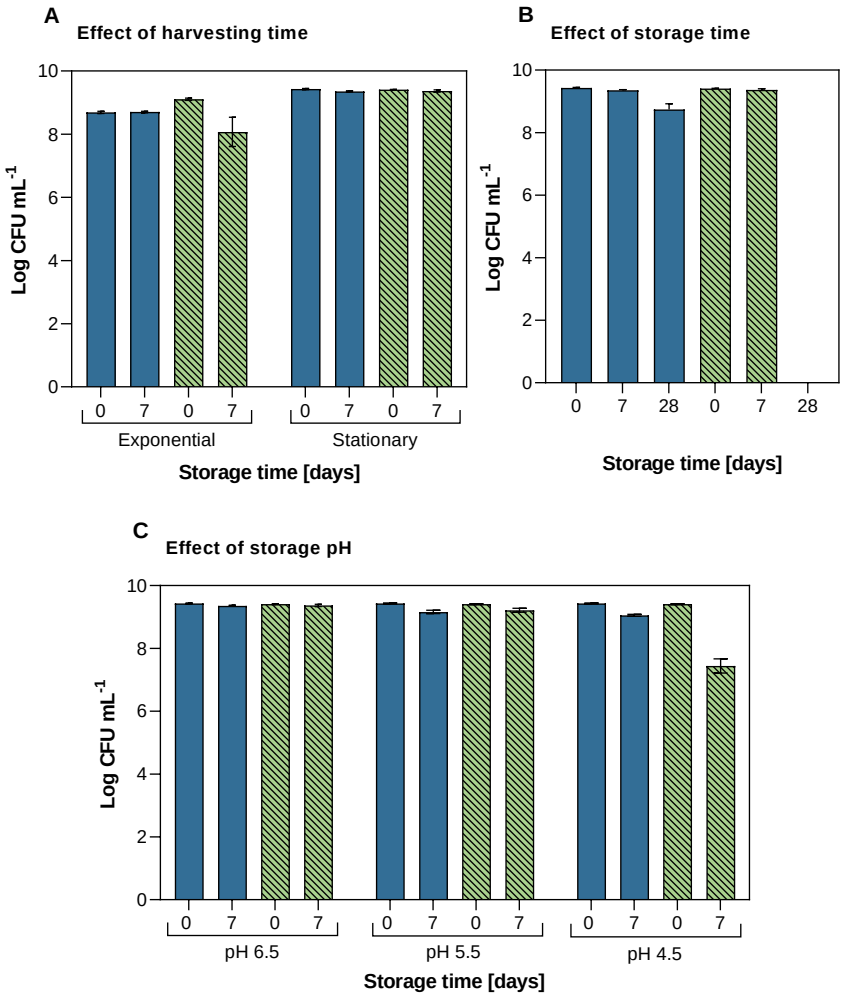


Figure 12: Survival of BB-12 (blue) and BB-46 (green) during short-term storage. Cells were stored under aerobic conditions for 7 to 28 days at 8–10°C in cultivation broth. Viability was assessed by CFU counts before and after storage. Each data point represents the mean of biological triplicates ± standard deviation. **A. Effect of harvesting time.** Strains were harvested in exponential and stationary phase, and stored for 7 days at pH 6.5. **B. Effect of storage time.** Strains were harvested in stationary phase and stored at pH 6.5 for 7 days or 28 days. **C. Effect of storage pH.** Strains were harvested in the stationary phase and stored at pH 6.5, pH 5.5, and pH 4.5 for 7 days (adapted from Figure 3 in **Paper III**).

4.3 Stress response in bifidobacteria

To overcome exposure to stressors, bifidobacteria are equipped with molecular defenses, which include not only constitutively active mechanisms, but also the ability to sense and efficiently respond to environmental perturbation with metabolic and physiological changes. The stress response in (probiotic) bifidobacteria has been first studied using classical methodologies, and more recently, through genomics, transcriptomics, and proteomics. Known phenotypic effects and mechanisms underlying the stress physiology in bifidobacteria are reviewed in **Paper II**, together with existing knowledge gaps and strategies for improving the robustness and stability of probiotic bifidobacteria.

This section provides an overview of well-studied stress response strategies employed by bifidobacteria. With a single exception, the presented strategies are supported by

physiological data or genetic engineering and have been observed in two or more *Bifidobacterium* strains. The list includes previous published evidence (as reviewed in **Paper II**) along with insights acquired from comparative studies on the stress physiology of BB-12 and BB-46 in this work (**Paper III, Paper IV**).

Defense strategies of bifidobacteria are categorized as: (i) general stress response, (ii) oxidative stress response, (iii) acid stress response (iv) acid and bile (acid) stress response and (v) bile (acid) stress response.

Mechanisms of the general stress response. While many physiological and metabolic responses are specific for the defense against a single stressor, other elements are shared across the response mechanisms of bifidobacteria to various stressors (as reviewed in **Paper II**).

Many stressors promote protein misfolding and denaturation (Begley et al., 2005; Sugimoto et al., 2008; Ventura et al., 2006), which is counteracted by the expression of chaperones and proteases (heat shock proteins) that secure proper protein folding though refolding or removal misfolded or denatured proteins (as reviewed in **Paper II**). The induction pattern of heat shock proteins varies between stressors (Zomer et al., 2009; Zomer & van Sinderen, 2010). Using *B. breve* UCC2003 as a representative strain, a model for the regulatory network of chaperones and proteases in bifidobacteria has been proposed based on genetic and transcriptional data (Zomer et al., 2009). In the present work, the binding motifs of transcriptional regulators involved in the regulatory network and their position in the genome were found not to be conserved across strains from different phylogenetic groups (**Paper V**). Therefore, the proposed model for the protein quality control regulatory network may only be valid for *Bifidobacterium* strains closely related to *B. breve* (**Paper V**). Indeed, differences in the induction pattern of heat shock proteins have been observed between strains upon exposure to the same stressor such as acid stress (as reviewed in **Paper II**). Moreover, a comparative study revealed that the chaperones ClpB and GrpE were consistently more expressed in BB-12 than in BB-46 (**Paper III**). Four chaperones (ClpB, DnaK, GroEL and ClpC) were among the hundred most expressed genes during exponential and stationary phase in BB-12; whereas, in BB-46, this held true only for GroEL (**Paper III**). Higher expression of chaperones in BB-12 might be attributed to enhanced maintenance of protein quality, thus explaining the higher robustness and stability of this strain.

Mediators of DNA repair and replication are commonly induced in *Bifidobacterium* strains in response to stress. Although this strategy has only been documented at the transcriptional and translational level, it applies to numerous strains and stressors (as reviewed in **Paper II**). The regulation of the DNA repair system (SOS response) overlaps with the protein quality control system in *B. breve* UCC2003 (Zomer et al., 2009). In BB-46, higher expression of genes involved in the maintenance of DNA repair in stationary phase may contribute to the strain's enhanced survival when harvested in this phase rather than in exponential phase (see section 4.2), as it prevents loss of genomic DNA integrity (**Paper III**).

Given that many defense mechanisms require energy in the form of ATP, an increased availability of ATP is another common element in the response of bifidobacteria to stressors. Different strategies have been observed across strains and stressors to improve the supply of ATP (as reviewed in **Paper II**), including:

i. slower growth and downsizing of the translation machinery (Guillaume et al., 2009; Talwalkar & Kailasapathy, 2003; Zomer et al., 2009),
ii. a metabolic shift towards acetate formation (Ruas-Madiedo et al., 2005; Ruiz, Gueimonde, et al., 2012; Sánchez et al., 2004, 2007; Talwalkar & Kailasapathy, 2003),
iii. the acceleration of carbon dissimilation, e.g., by increased activity and upregulation of glycolytic enzymes, such as Xfp (Collado & Sanz, 2007; Jin et al., 2015; Sánchez et al., 2004, 2005), and
iv. changes in carbon source preference and glycolytic activity (Noriega et al., 2004; Ruas-Madiedo et al., 2005).

Mechanisms of oxidative stress response. Oxidative stress is caused by the release of reactive oxygen species (ROS), including H_2O_2, superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($HO^{\cdot}$) under aerobic conditions. ROS can damage major macromolecules, such as proteins, DNA, and lipids (Ahn et al., 2001). In contrast to aerobic bacteria, anaerobic bacteria have fewer defenses against ROS.

Various O_2-scavenging enzymes that reduce O_2 to H_2O_2 or H_2O, and ROS-detoxifying enzymes that neutralize H_2O_2 and $O_2^{\cdot -}$ have been identified and characterized in *Bifidobacterium* strains (as reviewed in **Paper II)**. An interplay between these two classes of enzymes is crucial to prevent the accumulation of toxic H_2O_2. Indeed, excessive H_2O_2 production or its insufficient detoxification is thought to be one reason for high O_2-sensitivity of *Bifidobacterium* strains (De Vries & Stouthamer, 1969; Kawasaki et al., 2006; Shimamura et al., 1992; Shin & Park, 1997; Tanaka et al., 2018). In addition, not all *Bifidobacterium* strains are equipped with the same set of oxidative stress-associated genes, which could explain the observed variation in O_2-tolerance, as discussed in section 4.5 (**Paper V**).

In this work, several oxidative stress-associated genes were found to be relatively more expressed in the stable and robust BB-12 than in the stress-sensitive BB-46 strain in exponential and/or stationary phase (**Paper III**). Specifically, expression was relatively higher for genes encoding flavodoxin, bacterioferritin comigratory protein (BCP) (peroxiredoxin), thioredoxin (TrxA), thioredoxin-disulfide reductases (TrxR), (peptide)-L-methionine-(R/S) sulfoxide reductase (MsrAB), and DNA-binding protein from starved cells (Dps) (**Paper III**). Four of these genes (Dps > BCP > TrxR > TrxA) were among the hundred most expressed genes in BB-12 during stationary phase (**Paper III**). The high level of oxidative stress-associated genes in BB-12 may explain the strain's good survival under the applied aerobic storage conditions (section 4.1), as exemplified hereafter by BCP and MsrAB.

ROS can cause lipid peroxidation, which may alter the structure of the cell membrane (Lee et al., 2009). BCP-type peroxiredoxin has been found to preferably reduce linoleic acid hydroperoxide in *Escherichia coli* and *Heliobacter pylori* (Jeong et al., 2000; Wang et al., 2005). Elevated expression of its homolog in BB-12 might thus allow to prevent lipid peroxidation under aerobic conditions.

ROS can lead to oxidative damage of proteins and L-methionine and L-cysteine are the amino acids that are most susceptible to oxidation. Oxidation of the former results in the formation of methionine sulfoxide, which can be reduced back to methionine by MsrAB, indirectly contributing to ROS scavenging (Lee et al., 2009). Besides the relatively higher

expression of MsrAB, BB-12 seems to maintain a consistently high L-methionine concentration in its cytoplasm compared to BB-46 (**Paper III**). BB-12 showed an initial strong uptake of L-methionine, whereas almost no L-methionine was taken up by BB-46 (**Paper III**). Then, when L-methionine uptake decreased in stationary phase, genes associated with its biosynthesis from L-homoserine and L-cysteine, as well as genes encoding a transporter of methionine/methionine sulfoxide, were found to be upregulated in BB-12. Moreover, this was accompanied by an upregulation of MsrAB, as well as a slight increase in the expression of genes encoding TrxR and TrxA, which are required for the regeneration of reduced MsrAB (**Paper III**). A high L-methionine concentration in the cytoplasm of BB-12 might be attributed to enhanced activity of the ROS-scavenging L-methionine oxidation-reduction cycle and, consequently, the strong O_2-tolerance of the strain. Taken together, these results suggest that L-methionine addition to cultivation medium may promote the robustness and stability of some *Bifidobacterium* strains by fueling MsrAB activity, which may contribute to protein homeostasis and the supply of free amino acids under aerobic conditions (**Paper III**). However, this hypothesis remains to be validated.

Additional mechanisms against oxidative stress in bifidobacteria include changes to the cell membrane fatty acid profile, which is important also in the defense against acid and bile (acid) stress (as reviewed in **Paper II**). Examples of such changes are summarized hereafter.

i. Under partially aerobic conditions, two *B. longum* strains with relatively high O_2-tolerance showed an increase in short-chain and cyclopropane fatty acid content (Ahn et al., 2001). Cyclopropane fatty acids are considered highly chemically stable, which might contribute to a reduced susceptibility of the cell membrane under aerobic conditions (Grogan & Cronan, 1997).
ii. Upon lethal acid stress (pH 3.5), an increase in myristic acid (C14:0) content was observed in the cell membrane of an acid-resistant derivative of *B. longum* JDM301, but not in its acid-sensitive parental strain (Wei et al., 2019). It remains to be determined how a higher myristic acid content may contribute to increased acid stress tolerance.
iii. Upon bile stress, *B. animalis* subsp. *lactis* IPLA 4549 and a bile-resistant derivative showed an altered cell membrane fatty acid profile (Ruiz et al., 2007). The observed changes coincided with decreased membrane fluidity in both strains upon bile stress. In fact, the bile-resistant strain showed lower membrane fluidity than its parental strain even under non-stressed condition, most likely due to a lower ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) of the derivative (Ruiz et al., 2007).

The importance of the cell membrane fatty acid profile on the robustness and stability of *Bifidobacterium* strains was further demonstrated in **Paper IV**. First, the fatty acid profile of BB-12 and BB-46 differed considerably following lab-scale cultivation in CDM supplemented with 1 mL L^{-1} Tween® 80 as an exogenous fatty acid source (Figure 13) (**Paper III, Paper IV**). Specifically, the fatty acid profile of BB-12 was dominated by equal amounts of palmitic acid (C16:0) and oleic acid (C18:1); whereas oleic acid alone was predominant in the cell membrane of BB-46 (Figure 13A). The high content of oleic acid was attributed to a significantly higher UFA/SFA ratio in BB-46 compared to BB-12 (Figure 13B) (**Paper IV**). The observed fatty acid profile coincided with considerably higher

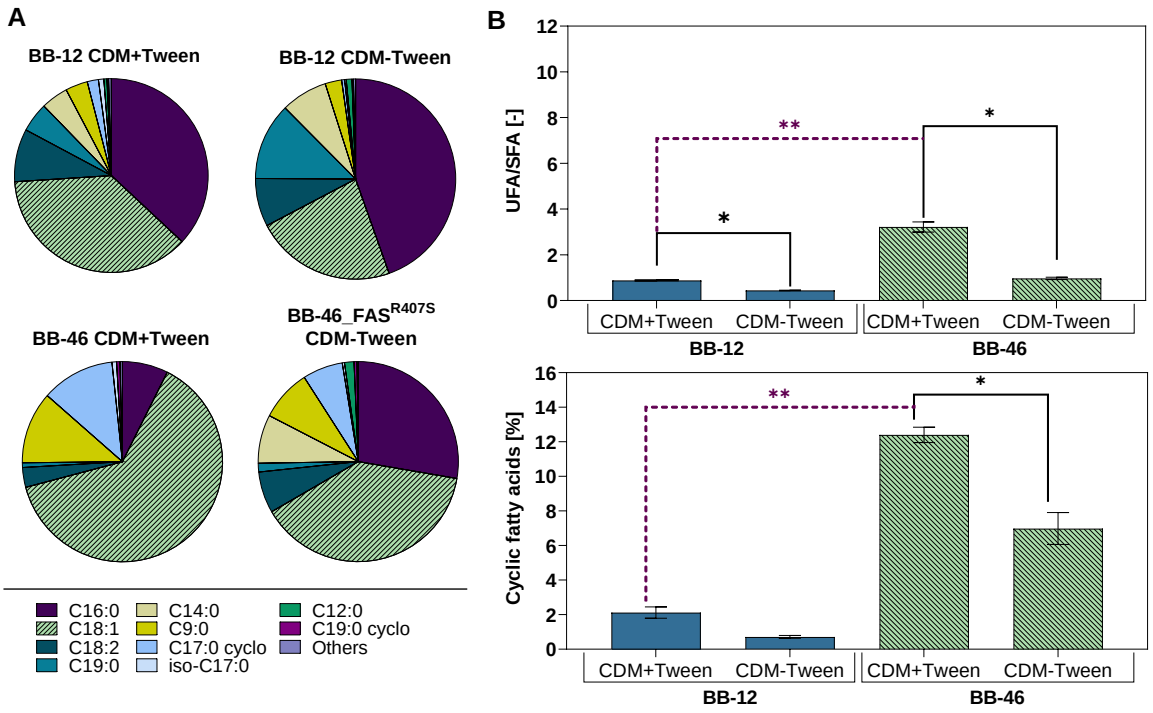


Figure 13: Fatty acid profile of BB-12 and BB-46 in CDM containing Tween® 80 (CDM+Tween), as well as of BB-12 and BB-46_FAS^{R407S} in CDM without Tween® 80 (CDM-Tween). The fatty acid profile was analyzed in stationary phase. **A. Content of individual fatty acids in the cell membrane.** Each value represents the mean of a triplicate. **B. Fatty acid profile characterization.** Each value represents the mean of a triplicate ± standard deviation. UFA/SFA: ratio of unsaturated to saturated fatty acids. The UFA/SFA ratio was calculated without considering cyclic fatty acids. Significant differences between means were tested by a multiple unpaired *t*-test with CDM-Ref serving as reference; *$p_{adj} < 0.05$, **$p_{adj} < 0.005$ (adapted from Figure 5 in **Paper IV**).

robustness and stability of BB-12 compared to BB-46 during storage under aerobic conditions for 28 days at pH 7.0 in peptone saline solution and at 2–4°C, as indicated by a viability loss of 1.0 ± 0.3 $\log_{10}$ CFU mL^{-1} for BB-12 and of 4.3 ± 1.7 $\log_{10}$ CFU mL^{-1} for BB-46 (**Paper IV**). Second, cultivation in CDM without Tween® 80 led to an increase in palmitic acid and a decrease in oleic acid content in the cell membrane of BB-12, lowering the UFA/SFA ratio to 0.4 ± 0.0 (Figure 13B), but without altering the strain's robustness and stability (0.9 ± 0.5 $\log_{10}$ loss). The maximum specific growth rate of BB-12 was slightly higher in CDM without Tween® 80 ($\mu = 0.52 \pm 0.02$ h^{-1}) than in the reference CDM ($\mu = 0.46 \pm 0.00$ h^{-1}) (**Paper IV**). In contrast to BB-12, BB-46 could not grow in the absence of an exogenous fatty acid source (**Paper IV**). However, when BB-46 cells were inoculated in CDM without Tween® 80, a variant carrying a single nucleotide polymorphism (SNP) in a gene encoding the microbial type I fatty acid synthase (FAS) (**Paper IV**) emerged through natural selection. FAS is essential for *de novo* fatty acid biosynthesis (Schweizer & Hofmann, 2004). The SNP resulted in the substitution of arginine for serine at position 407 of FAS and the variant was termed BB-46_FAS^{R407S} (**Paper IV**). The observed mutation was likely attributed to a newly acquired capacity for *de novo* fatty acid biosynthesis (**Paper IV**).

Interestingly, BB-46_FAS^{R407S} demonstrated improved robustness and stability (1.4 ± 0.0 $\log_{10}$ loss), as well as significantly more palmitic acid and less oleic acids compared to BB-46 cultivated in CDM (Figure 13A). Overall, the fatty acid composition of the cell membrane

of BB-46_FAS^{R407S} was more similar to that of BB-12 with a UFA/SFA ratio of around 1 (Figure 13B). The growth rate, end fermentation products, cell morphology, and hydrophobicity did not differ considerably between BB-46 cultivated in CDM and BB-46_FAS^{R407S} cultivated in CDM without Tween® 80 (**Paper IV**). Only the amino acid profile differed between the two strains (**Paper IV**), most likely because both fatty acid and amino acid biosynthesis use pyruvate as precursor and NADPH as reducing agent (**Paper IV**). Taken together, the improved robustness and stability of BB-46_FAS^{R407S} compared to BB-46 cultivated in CDM was likely the result of altered cell membrane fatty acid composition.

In agreement with the above results, the cell membrane of H_2O_2-resistant *B. animalis* subsp. *lactis* BL-04 was reported to have higher palmitic acid and lower oleic acid content, together with a lower UFA/SFA ratio than the genetically closely related H_2O_2-sensitive *B. animalis* subsp. *lactis* DSM 10140 when grown in medium with Tween® 80 (Oberg et al., 2011). The higher H_2O_2 tolerance was linked to higher cyclic fatty acid content in the cell membrane of BL-04 (Oberg et al., 2013). However, this hypothesis conflicts with the finding described in this thesis, whereby the cell membrane has a higher cyclic acid content in the stress-sensitive BB-46 than in BB-12 (Figure 13B). Moreover, when grown in the absence of any fatty acid source, the stress tolerance of DSM 10140 actually increased (Oberg et al., 2013), coinciding with an increased palmitic acid content but a decreased oleic acid content (Oberg et al., 2013). Thus, it is possible that a lower UFA/SFA ratio in the absence of Tween® 80 might have boosted H_2O_2 tolerance of DSM 10140.

Mechanisms of acid stress response. Maintaining the intracellular pH (pH homeostasis) is essential for a bacterial cell as it ensures a proton motive force across the cell membrane, which drives various transport processes. The presence of weak acids in the environment can challenge the maintenance of this proton motive force. As discussed in section 3.5, weak acids are partially protonated across a wide range of pH values and can passively diffuse across the cell membrane. Inside the cell, the weak acid may become deprotonated due to a higher pH, which results in acidification of the cytoplasm. In contrast, dissociated strong acids cannot diffuse into the cell, and their toxicity is associated with damage to biomolecules on the cell surface (Beales, 2004). Moreover, as they lower the external pH, they cause a steeper pH gradient across the membrane, which promotes proton permeability and cytoplasmic acidification (Beales, 2004).

Bifidobacteria address acid stress through the intracellular accumulation of polyphosphates, which are linear polymers of orthophosphate residues linked by phosphoanhydride bonds (as reviewed in **Paper II**). Polyphosphate kinase (EC 2.7.4.1), which catalyzes the formation of the link between a polyphosphate chain and a phosphate ion, was found to be upregulated upon exposure to sublethal acid stress in *B. longum* BBMN68 (Jin et al., 2012). Moreover, *B. scardovi* BAA-773 cells that accumulated polyphosphate showed increased acid resistance (Qian et al., 2011). Polyphosphates might contribute to acid tolerance by functioning as a buffer to minimize pH changes, although they may exert also additional functions (Anand & Aoyagi, 2019; Mullan et al., 2002; Schröder & Müller, 1999). BB-12 and BB-46 harbored similar levels of polyphosphate granules when cultivated in pH-controlled CDM (**Paper III**), indicating that polyphosphate accumulation was an unlikely source of divergent robustness and stability between these two strains.

Increased peptidoglycan biosynthesis has been described as another defense mechanism of bifidobacteria against acid stress. Both exposure and adaptation to acid stress have been reported to promote peptidoglycan biosynthesis (Jin et al., 2015; Wei et al., 2019; Yang et al., 2015), which might compensate for damaged peptidoglycan or allow for adjustments to its fine structure (as concluded in **Paper II**).

Shared mechanisms between acid and bile (acid) stress response. In the intestine, bifidobacteria are exposed to bile, whose main organic constituent are bile salts, a term that is often used synonymous with bile acids (Begley et al., 2005). Before their secretion from the liver, bile acids are conjugated with either glycine or taurine (Begley et al., 2005). Under physiological pH, conjugated bile acids are almost fully ionized as bile salts (Hofmann & Hagey, 2008). Bile salts function as biological detergents and exhibit strong antimicrobial properties (Begley et al., 2005). The growth inhibition of bile salts has been found to be concentration-dependent (Kurdi et al., 2006). The toxicity of bile salts can be attributed to their membrane-damaging effect and unconjugated bile salts share the mechanism of growth inhibition with that of weak acids (Kurdi et al., 2003, 2006). In agreement with the overlapping inhibition mechanism of both stressors, a considerable number of strategies are shared between acid and bile acid stress response in bifidobacteria (as reviewed in **Paper II**).

First, the proton-translocating F_1F_0-ATPase contributes to the maintenance of pH homeostasis in *B. animalis* and *B. longum* strains upon exposure to both stressors by actively pumping protons out of the cytoplasm (Jin et al., 2015; Matsumoto et al., 2004; Sánchez et al., 2006; Ventura et al., 2004). In the present work, transcriptomic analysis revealed that most subunits of the F_1F_0-ATPase were higher expressed in BB-12 than in BB-46 during stationary phase, which might contribute to the enhanced acid tolerance of BB-12 compared to BB-46 during short-term storage at pH 4.5 (section 4.1, **Paper III**).

Second, some *Bifidobacterium* strains display increased expression and activity of enzymes that stimulate the consumption of intracellular protons and the formation of CO_2. The latter can be converted to bicarbonate, and thereby buffer the cytoplasmic pH (as reviewed in **Paper II**). Some *Bifidobacterium* strains, particularly *B. adolescentis*, can release γ-aminobutyrate, whose production is coupled to the consumption of an intracellular proton and the formation of CO_2 (Barrett et al., 2012; Duranti et al., 2020; Yunes et al., 2016). Acid stress has been found to induce the expression of genes responsible for γ-aminobutyrate biosynthesis in *B. dentium* (Ventura et al., 2009). In certain strains, exposure to bile acid and acid stress promotes the decarboxylation of the organic acid oxalate via oxalyl-CoA, which consumes an intracellular proton (Sánchez et al., 2007; Turroni et al., 2010; Ventura et al., 2009). The two enzymes responsible for oxalate degradation are formyl-CoA transferase and oxalyl-CoA decarboxylase. In this work, these two enzymes were found to be among the ten most expressed genes in BB-12 during stationary phase when cultivated in CDM, even in medium devoid of oxalate (**Paper III**). The ability to degrade oxalate may benefit acid tolerance in BB-12 when oxalate is actually present, as well as add to the strain's probiotic effect (Turroni et al., 2010).

Third, exopolysaccharides and biofilms formed by *Bifidobacterium* strains were found to affect tolerance towards bile and acid stress, and their levels were documented to change upon exposure to these stressors (Alp & Aslim, 2010; Fanning et al., 2012; Jiang et al., 2015; Jin et al., 2012; Kelly et al., 2020; Ruas-Madiedo et al., 2009; Yang et al., 2015). Generally,

exopolysaccharides and biofilm are thought to offer physical protection against acids (Alp & Aslim, 2010; Fanning et al., 2012; Jin et al., 2012; Kelly et al., 2020; Yang et al., 2015). However, also decreased exopolysaccharides production seems to have a beneficial effect by promoting cell aggregation (Jiang et al., 2015).

Mechanisms of bile (acid) stress response. Specific mechanisms to cope with bile stress have been reported in bifidobacteria. One such strategy is the active transport of bile salts out of the cell and various putative transporter have been identified, including the multidrug resistance transporters BetA (An et al., 2014; Gueimonde et al., 2009; Ruiz et al., 2012; Ruiz et al., 2012), BbmAB (Gueimonde et al., 2009), BmrAB (Xu et al., 2019), and Ctr (homolog of BbmR) (Margolles et al., 2005; Price et al., 2006). The contribution of these transporters to bile efflux was confirmed by gene upregulation following exposure to bile (Gueimonde et al., 2009), through gene knockouts (Ruiz et al., 2012) or heterologous gene expression (Gueimonde et al., 2009; Price et al., 2006; Ruiz et al., 2012).

While the importance of bile salt efflux is evident, the function of the bile salt hydrolase (BSH; EC 3.5.1.24), which catalyzes the deconjugation of bile salts, remains unclear (as reviewed in **Paper II**). The main counterargument for a contribution of BSH activity to increased bile salt resistance in bifidobacteria is that deconjugated bile salts are more toxic than their conjugated counterparts (Ruiz et al., 2013). Nevertheless, intracellular BSH activity might be beneficial for the cells, as deconjugated bile acids may recapture co-transported protons in the cytoplasm, and thus provide an advantage over cells lacking BSH activity (Begley et al., 2005).

Bifidobacteria further induce molecular factors involved in protein synthesis upon bile (acid) stress, such as ribosome-associated protein Y (An et al., 2014; Ruiz et al., 2012) and oligo- and dipeptide transporters (Ruiz et al., 2009). This response may secure activity and fidelity of protein synthesis under bile acid stress (as concluded in **Paper II**).

In addition, bile acid stress or adaptation to it induces changes to the cell surface properties of *Bifidobacterium* strains, including hydrophobicity, adhesion, and aggregation (An et al., 2014; Gomez Zavaglia et al., 2002; Gueimonde et al., 2005, 2007; Kociubinski et al., 2002). These modifications were found to vary between strains as well as in relation to bile components as opposed to whole bile (e.g., cholate and ox-gall) (Gomez Zavaglia et al., 2002; Gueimonde et al., 2005, 2007; Kociubinski et al., 2002). Besides a putative positive effect on bile tolerance, changes to cell surface properties may also favor gut colonization (as concluded in **Paper II**).

Cell surface properties of *Bifidobacterium* strains seem to also impact the tolerance to other stressors (as reviewed in **Paper II**). For example, exposure to oxidative stress lowered dramatically the cell surface hydrophobicity of *B. longum* BBMN68 (Zuo et al., 2018). In this work, cell surface hydrophobicity differed substantially between the stable and robust BB-12 and the stress-sensitive BB-46 when cultivated in CDM (**Paper III**). Specifically, BB-12 displayed a hydrophobic cell surface, whereas BB-46 had a hydrophilic one (**Paper III**). Concurrently, sedimentation of BB-12 cells was observed during short-term storage, whereas BB-46 cells maintained an even distribution throughout the suspension. It is conceivable that the strong hydrophobicity of BB-12 cells may have promoted autoaggregation and, consequently, sedimentation, thereby diminishing exposure to

stressors such as O_2 (**Paper III**). Thus, greater hydrophobicity of the BB-12 cell surface may partly explain the strain's improved survival compared to BB-46.

Knowledge gaps. In spite of increasing knowledge on the stress response in bifidobacteria over the last decades, reviewing the available literature revealed also some important gaps in the field (**Paper II**). While the stress response of bifidobacteria to some stressors, such as O_2 and acids, has been widely investigated, the response to other stressors, such as high osmolality and low temperature, remains largely uncharted. Moreover, the stress response of a few strains, such as *B. breve* UCC2003 and *B. longum* NCC27005, has received a lot of attention, but has not been followed by validation in other strains or species. This is of particular importance given that stress tolerance by bifidobacteria varies considerably across species and strains. Hence, results obtained with certain species or strains cannot be simply extrapolated to other members of the genus.

The rise of omics technologies has provided new insights on the stress response; however, several hypotheses based on these analyses remain to be validated in physiological studies. Vice versa, only a few molecular players of the stress response in bifidobacteria have been subjected to comprehensive functional studies, which is essential for a correct interpretation of omics data. Finally, the comparison of existing results is hampered by the application of different conditions during cultivation, stress treatment, and viability assessment (as reviewed in **Paper II**).

In this work, the comparison between BB-12 and BB-46 revealed a stronger expression of several stress-associated genes in BB-12 than in BB-46 (**Paper III**). Accordingly, BB-12 seems to be intrinsically better equipped against diverse stressors, which may enable an instant response, minimize cellular damage, and ensure higher survival. The robustness and stability of other *Bifidobacterium* strains such as BB-46 may be improved by inducing the expression of stress-associated genes found to be upregulated in BB-12. Moreover, these stress-associated genes represent candidate biomarkers, whose expression level could be used to predict the robustness and stability of *Bifidobacterium* strains. Strong robustness and stability of BB-12 may be further explained by a relatively lower UFA/SFA ratio in the cell membrane, as well as elevated cell surface hydrophobicity.

4.4 Importance of medium composition

Besides the choice of process settings, such as pH, temperature or mixing rate, the formulation of a suitable medium is crucial when developing or optimizing industrial cultivation processes (section 2.3). Indeed, previous studies have demonstrated that medium composition can considerably influence the growth and physiology of probiotic bacteria. Growth and acid tolerance of several *Bifidobacterium* strains were found to differ depending on the selected propagation medium (Mättö et al., 2004). Moreover, propagation of probiotic *Propionibacterium freudenreichii* in hyper-concentrated cheese whey medium induced tolerance to various stressors, thereby resulting in improved robustness and stability (Huang et al., 2016).

To investigate the effect of different medium components on the performance of BB-12 and BB-46, lab-scale batch cultivations at pH 6.5 in different media (Table 4) were performed (**Paper IV**).

Table 4: Properties of the five media applied for lab-scale cultivation of BB-12 and BB-46 to test their effect on growth, robustness, and stability. CDM: chemically defined medium, MRS: De Man, Rogosa and Sharpe, PABA: *para*-aminobenzoic acid.

	Original CDM (reference)	CDM with elevated vitamin concentrations	CDM without Tween® 80	CDM with casein hydrolysate	MRS medium
Nitrogen source	500 mg L^{-1} cysteine, 40 mg L^{-1} of each additional proteinogenic amino acid	See original CDM	See original CDM	Casein hydrolysate from bovine milk; higher content of free amino acids than in reference medium except for glutamine and glycine	Undefined[1)]; higher content of free amino acids than in reference medium except for glutamine
Fatty acid source	1 mL L^{-1} Tween® 80	See original CDM	None	See original CDM	1 mL L^{-1} Tween® 80 and additional undefined amounts[1)]
Vitamin source	0.2–10 mg L^{-1} pantethine, nicotinic acid, pantothenate, pyridoxal, menaquinone-4, PABA, nicotinamide, riboflavin, cobalamin, dl-6,8-thioctic acid, thiamine, biotin, and folic acid[2)]	Twice the amount of PABA, calcium pantothenate, biotin, folic acid, nicotinamide, pyridoxal-HCl, riboflavin, thiamine-HCl, cobalamin and dl-6,8-thioctic acid than in original CDM	See original CDM	See original CDM	Undefined[1)]

[1)] Supplied by yeast extract, enzymatic digest of animal tissue, and beef extract. [2)] See **Paper I** for the exact concentrations.

The newly-developed CDM (section 3.4) was used as the reference condition. Having a defined composition, it was possible to remove, increase or exchange individual medium compounds. Overall, the effect of the nitrogen source, exogenous fatty acid supply, and elevated vitamin supply was investigated in media formulated based on the newly-developed CDM (Table 4). As a comparison, the two strains were cultivated also in De Man, Rogosa and Sharpe (MRS) medium, which contained undefined complex compounds (Table 4) and simulated the medium traditionally used for industrial cultivations. The same gas phase composition of 80% N_2 and 20% CO_2 was applied for all cultivations.

The effect of medium composition on the metabolism and physiology of BB-12 and BB-46 was assessed in terms of growth, metabolite production, amino acid utilization, cell envelope characteristics, robustness, and stability across five media (**Paper IV**). Robustness and stability were assessed by determining survival after short-term storage (see section 4.1). The cells were harvested in stationary phase and stored at 2–4°C in peptone saline solution (pH = 7.0) for 28 days under aerobic conditions.

No considerable differences in the analyzed characteristics were detected when BB-46 was cultivated in CDM with an elevated vitamin concentration compared to reference CDM (**Paper IV**). In contrast, BB-12 grew slower under this condition ($\mu = 0.42 \pm 0.01$ h^{-1}) and exhibited poorer survival with a viability loss of 1.8 ± 0.1 $\log_{10}$ CFU mL^{-1} compared to the reference CDM ($\mu = 0.46 \pm 0.01$, 1.0 ± 0.3 $\log_{10}$ loss). This result suggested that the vitamin concentration in the reference CDM was not limiting growth, biomass yield or the defense mechanisms employed by the two strains, and that a higher vitamin concentration can even be obstructive.

The effect of cultivating BB-12 and BB-46 in the absence of an exogenous fatty acid source has been already discussed in section 4.3. Briefly, in the absence of Tween® 80, BB-12 showed higher maximum specific growth rate and a lower UFA/SFA ratio (**Paper IV**) than in its presence. No effect on the robustness and stability of BB-12 was observed. Moreover, the inoculation of CDM lacking Tween® 80 with BB-46 cells led to the natural selection of the BB-46_FAS^{R407S} variant, whose fatty acid profile was more similar to that of BB-12, while its robustness and stability were better than that of the parental strain (**Paper IV**).

In complex MRS medium (Table 4), BB-12 and BB-46 showed poor robustness and stability, with a viability loss of 6.2 ± 2.0 $\log_{10}$ CFU mL^{-1} and 7.0 ± 1.0 $\log_{10}$ CFU mL^{-1}, respectively (**Paper IV**). Moreover, additional metabolic and physiological characteristics of BB-12 and BB-46 differed when they were cultivated in MRS medium, as compared to the reference CDM.

First, BB-12 reached a maximum specific growth rate of 0.53 ± 0.01 h^{-1} in MRS medium, which exceeded that in the reference CDM (0.46 ± 0.00 h^{-1}). In contrast, the maximum specific growth rate of BB-46 in MRS coincided with that in reference CDM ($\mu = 0.35 \pm 0.02$ h^{-1}), along with a longer lag phase and comparatively short exponential phase (**Paper IV**). Taken together, these results highlight that the supply of complex substrates can, but does not always, correlate with high cultivation efficiency of *Bifidobacterium* strains.

Second, a significant increase in lactate formation and decrease in formate production was observed for BB-46 when cultivated in MRS medium compared to reference CDM (**Paper IV**). Instead, the fermentation end product profile of BB-12 did not differ between cells cultivated in MRS medium or reference CDM (**Paper IV**).

Third, several differences were detected in the cell membrane fatty acid composition of BB-12 and BB-46 when grown in MRS medium compared to reference CDM (**Paper IV**). These changes resulted in a higher UFA/SFA ratio in BB-12 (from 0.8 ± 0.0 to 1.2 ± 0.0), but especially in BB-46 (3.2 ± 0.2 to 8.0 ± 0.7). The difference observed in the fatty acid profile of BB-12 and BB-46 between MRS medium and reference CDM, both of which contained 1 mL L^{-1} Tween® 80, might be due to an undefined supply of fatty acids in the complex MRS medium (Table 4). Moreover, given that the BB-46_FAS^{R407S} variant displayed both a lower UFA/SFA ratio and improved survival during short-term storage (section 4.3), the increased UFA/SFA in the cell membrane of BB-12 and BB-46 cells cultivated in MRS medium might contribute to the poor robustness and stability of both strains under this condition. However, the increased UFA/SFA ratio in BB-12 during cultivation in MRS medium was similar to that of the BB-46_FAS^{R407S} variant (1.2 ± 0.0), which showed better survival during short-term storage (**Paper IV**). Therefore, the poor robustness and stability of BB-12 in MRS medium may be further ascribed to additional cellular changes, as proposed hereafter.

BB-12 showed a considerably larger size when cultivated in MRS medium than in other media (**Paper IV**). Previous studies on *lactobacilli* have suggested that shorter cells show better stress tolerance due to exposure of a smaller cell surface area to environmental stressors (Rajab et al., 2020; Senz et al., 2015). Conversely, the increased cell surface of BB-12 in MRS medium might result in lower tolerance to stressors, which may impact membrane integrity and survival (**Paper IV**). This hypothesis can only hold true if the mechanism against environmental stressors does not increase proportionally to cell size (**Paper IV**).

In semi-CDM with casein hydrolysate (Table 4), BB-12 and BB-46 demonstrated a higher maximum specific growth rate (0.52 ± 0.05 h^{-1} and 0.42 ± 0.02 h^{-1}) than in reference CDM (**Paper IV**). As mentioned in section 3.4, these results suggest that the supply of peptides can have a growth-promoting effect on *Bifidobacterium* strains, which is in agreement with previous evidence (Zhang et al., 2020).

The high maximum growth rate of BB-46 in semi-CDM with casein hydrolysate coincided with the lowest survival of the strain (7.4 ± 0.9 $\log_{10}$ loss) during short-term storage compared to all other tested media (**Paper IV**). Moreover, as in complex MRS medium, the strain demonstrated a significant increase in lactate formation and reduced formate production compared to reference CDM (**Paper IV**). Taken together, it seems that a complex nitrogen source affects metabolite production by BB-46. It remains to be determined what the underlying mechanism is and whether the fermentation end product profile contributes to the poor robustness and stability under these conditions.

Overall, the hydrophobicity of BB-12 and BB-46 did not change significantly across the tested media, and BB-12 displayed a consistently more hydrophobic cell surface than BB-46 (**Paper IV**). However, based on the hydrophobicity assay used in this study, hydrophobic moieties on the cell surface of BB-12 are located deeper in the cell wall when the strain is cultivated in semi-CDM with casein hydrolysate than in other media (**Paper IV**).

To sum up, the presented results highlight the large influence exerted by cultivation medium composition on the metabolism and physiology of *Bifidobacterium* strains, including their growth rate, robustness, and stability. Because these are all key parameters in industrial

production, medium formulation deserves particular attention when optimizing the production of probiotic bifidobacteria.

4.5 Prevalence of stress-associated genes across *Bifidobacterium* strains

High-throughput genome sequencing has considerably advanced our understanding of the genetics of bifidobacteria. Reviewing existing knowledge on the stress response of bifidobacteria sparked the two following questions: "How well conserved are previously described stress-associated genes across the genus?" and "is the presence/absence of stress-associated genes in the genome of a *Bifidobacterium* strain indicative of its tolerance towards stressors?". The varying stress physiology of bifidobacteria may, at least in parts, be ascribed to a diverse gene content.

To address the above questions, the presence of 76 previously described stress-associated genes was assessed in 171 genome-sequenced *Bifidobacterium* strains, including BB-12 and BB-46 (**Paper V**). The protein sequence of each query gene was extracted from the genome sequence of the *Bifidobacterium* strain, whose stress response it had been suggested to mediate. All stress-associated genes were grouped into the following six categories: i) protein quality and DNA repair systems (heat stress), ii) oxidative stress, iii) acid stress, iv) bile stress, v) organic solvent stress, and vi) putative regulators of stress response. Due to limited information on the response of bifidobacteria to other stressors such as osmotic stress, no other categories were included. Genomic analysis provided information on the presence/absence of each gene in the genome of the analyzed strains, as well as the sequence percent identity and sequence coverage of homologs to the query gene. A cutoff of ≥ 40% sequence identity over at least 70% of the query sequence was selected for the analysis. The obtained stress-associated gene profiles were interpreted in relation to available phenotypic data on the stress tolerance of *Bifidobacterium* strains, as well as evidence of the molecular mechanisms underlying the robustness and stability of individual strains (**Paper V**).

Most variations regarding the presence of stress-associated genes were observed between strains of different phylogenetic groups and species, whereas relatively little variation was detected among strains of the same species. As a result, the outcomes were aggregated by species (Figure 14).

Genes involved in protein quality control and DNA repair were highly conserved in the *Bifidobacterium* genus (Figure 14). However, as mentioned in section 4.3, the regulation of these systems seems to differ among phylogenetic groups (**Paper V**).

In contrast, oxidative stress-associated genes were less conserved across species (Figure 14) (**Paper V**). Only strains isolated from the insect gut, including those of *B. actinocoloniiforme*, *B. asteroides*, *B. coryneforme*, and *B. indicum*, as well as *B. subtile* were confirmed to harbor genes encoding an electron transport chain (Milani et al., 2014). Additionally, these strains harbored a homolog of a superoxide dismutase gene from *B. xylocopae* subsp. nov. XV2 (Alberoni et al., 2019); whereas a homolog of a catalase gene from the same organism was only identified in a subset of these strains.

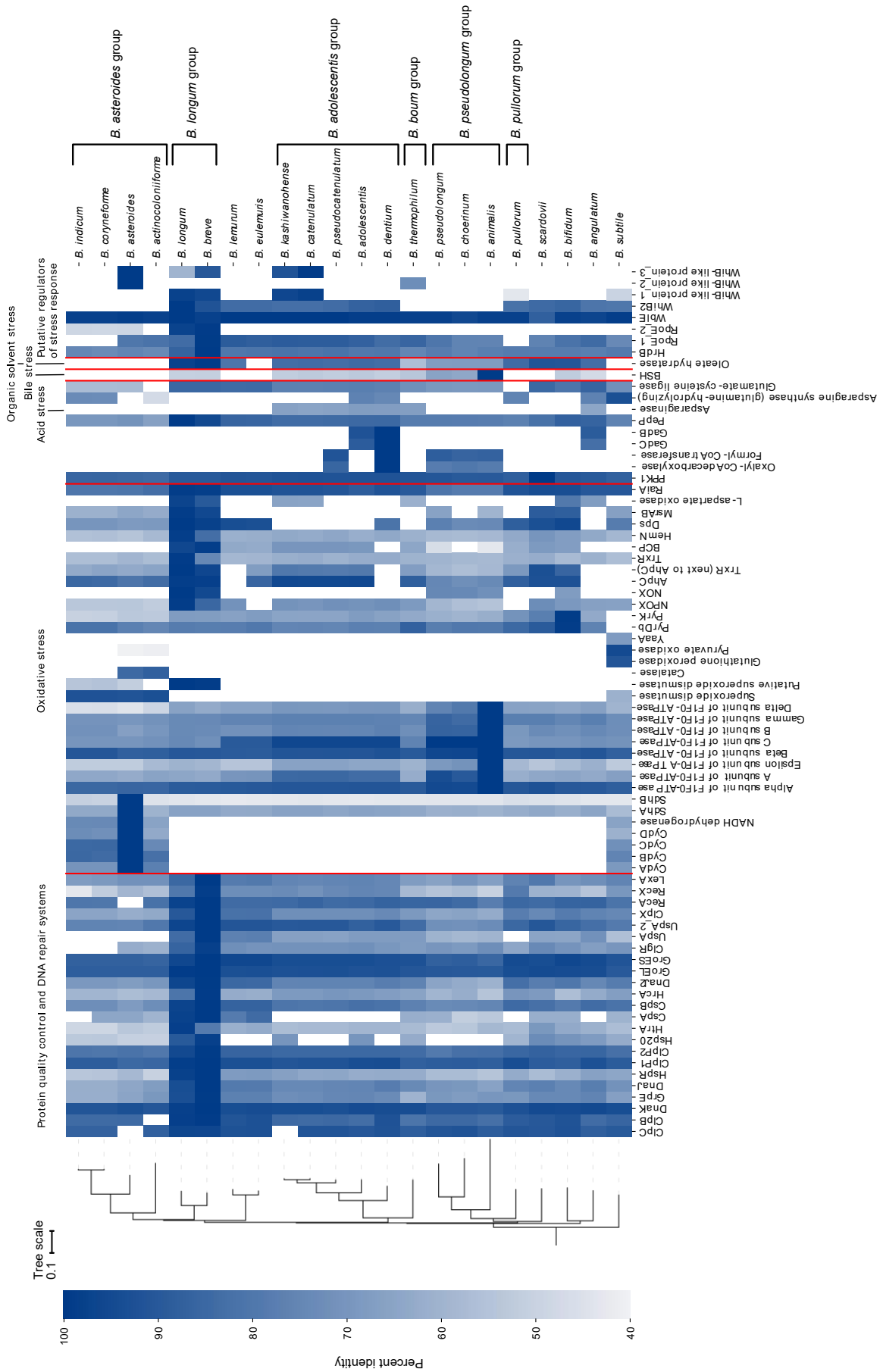

B. asteroides group
B. longum group
B. adolescentis group
B. boum group
B. pseudolongum group
B. pullorum group
B. indicum
B. coryneforme
B. asteroides
B. actinocoloniiforme
B. longum
B. breve
B. lemurum
B. eulemuris
B. kashiwanohense
B. catenulatum
B. pseudocatenulatum
B. adolescentis
B. dentium
B. thermophilum
B. pseudolongum
B. choerinum
B. animalis
B. pullorum
B. scardovii
B. bifidum
B. angulatum
B. subtile
Organic solvent stress
Bile stress
Acid stress
Putative regulators of stress response
Oxidative stress
Protein quality control and DNA repair systems
WhiB-like protein_1
WhiB-like protein_2
WhiB-like protein_3
WhiB2
WblE
RpoE_2
RpoE_1
HrdB
Oleate hydratase
BSH
Glutamate- cystein ligase
Asparagine synthase (glutamine- hydrolyzing)
Asparaginase
FepP
GadB
GadC
Formyl- CoA Transferase
Oxalyl- CoA decarboxylase
PPK1
RalA
L- aspartate oxidase
MsrAB
Dps
HemN
BCP
TrxR
TrxR (next to AhpC)
AhpC
NOX
NPOX
PyrK
PyrDb
YaaA
Pyruvate oxidase
Glutathione peroxidase
Catalase
Putative superoxide dismutase
Superoxide dismutase
Delta subunit of F1F0- ATPase
Gamma subunit of F1F0- ATPase
B subunit of F1F0- ATPase
C subunit of F1F0-ATPase
Beta subunit of F1F0- ATPase
Epsilon subunit of F1F0-A TPase
A subunit of F1F0-ATPase
Alpha subunit of F1F0-ATPase
SdhB
SdhA
NADH dehydrogenase
CydD
CydC
CydB
CydA
LexA
RecX
RecA
ClpX
UspA_2
UspA
ClgR
GroES
GroEL
DnaJ2
HrcA
CspB
CspA
HtrA
Hsp20
ClpP2
ClpP1
HspR
DnaJ
GrpE
DnaK
ClpB
ClpC
Tree scale
0.1
Percent identity
100
90
80
70
60
50
40

Figure 14: Heat map presenting the median sequence identity of the best hit for 76 stress-associated gene products in 22 *Bifidobacterium* species, including 171 *Bifidobacterium* strains. The analyzed *Bifidobacterium* species are members of six previously defined phylogenetic groups (Sun et al., 2015; Ventura et al., 2006). For each stress-associated gene, a query protein sequence was extracted from the genome of a strain, in which it was proposed to participate in stress responses. Homologs of stress-associated gene products across the 22 species were identified using DIAMOND BLASTp (E-value: 0.001, sequence identity cutoff: 40%, coverage cutoff: 70%). The maximum likelihood phylogeny tree was constructed using CLC Genomics (adapted from Figure 1 in **Paper V** under the CC BY 4.0 license).

Most *B. adolescentis* and all *B. angulatum* strains missed several ROS-detoxifying enzymes (Table 5, **Paper V**). The lack of these enzymes might explain the exceptionally high O_2 sensitivity reported for these strains (Scardovi & Crociani, 1974; Shimamura et al., 1992; Shin & Park, 1997).

Interestingly, homologs of all oxidative stress-associated genes listed in Table 5, except the gene encoding Dps, were also missing in the three studied strains of *B. dentium* (**Paper V**). *B. dentium*, which has been first isolated from the oral cavity of humans (caries) (Scardovi & Crociani, 1974), has not been described as exceptionally O_2-sensitive, suggesting that Dps plays an essential role in O_2-tolerance by bifidobacteria (**Paper V**).

Homologs of several oxidative stress-associated genes were found to show low sequence identity across species (Figure 14). One such example is the gene predicted to encode BCP-type peroxidase. Multiple sequence alignment of all identified BCP homologs revealed that BCP was a 1-Cys peroxiredoxin in *B. animalis* strains, but a 2-Cys peroxiredoxin in all other *Bifidobacterium* strains analyzed. While 1-Cys and 2-Cys peroxiredoxins differ in terms of their regeneration mechanism (Nelson et al., 2011), the functional implication of this finding remains unknown.

Table 5: Set of oxidative stress-associated genes in *B. angulatum* and *B. adolescentis* strains.

Protein Name	Function	Absent in
AhpC and TrxR (replacing the function of AhpF in bifidobacteria)	Peroxidase	*B. angulatum*: 2 of 2 strains *B. adolescentis*: 5 of 8 strains
Bacterioferritin comigratory protein (BCP)	Peroxidase	*B. angulatum*: 2 of 2 strains *B. adolescentis*: 3 of 8 strains
DNA-binding protein from starved cells (Dps)	Protection of DNA against ROS and scavenging of Fe^{2+} that would otherwise react in the Fenton reaction and form $OH^{\cdot}$	*B. angulatum*: 2 of 2 strains *B. adolescentis*: 8 of 8 strains
Peptide-methionine sulfoxide reductase MsrAB	Reduces methionine sulfoxide generated from the oxidation of methionine) back to methionine and thereby scavenges ROS	*B. angulatum*: 2 of 2 strains *B. adolescentis*: 8 of 8 strains
NADH oxidase (H_2O forming)	Reduction of oxygen to water using NADH as electron donor	*B. angulatum*: 2 of 2 strains *B. adolescentis*: 8 of 8 strains

Almost identical O_2-scavenging and ROS-detoxifying enzymes were detected in the genomes of O_2-tolerant strains of *B. animalis* subsp. *lactis* and O_2-sensitive strains *B. longum*, *B. breve*, and *B. bifidum* (**Paper V**). The disparity in O_2 tolerance among these strains might be explained by variations in the sequence of oxidative stress-associated genes, as well as their relative expression levels. This is in agreement with the findings from a previous study on the H_2O_2-forming NADPH oxidase (NPOX) from *B. infantis* ATCC 15697, which was linked to H_2O_2 accumulation and thereby to O_2-hypersensitivity (Tanaka et al., 2018). Heterologous expression of the gene encoding NPOX in the O_2-tolerant strain *B. minimum* DSM 20102, which possesses an NPOX homolog itself (**Paper V**), led to growth inhibition at O_2 concentrations above 10% (Tanaka et al., 2018). These results suggest that in wild type *B. minimum* DSM 20102, NPOX is most likely not expressed at high levels and that the activity of H_2O_2-detoxifying enzymes is sufficient to avoid H_2O_2 accumulation under aerobic conditions. Moreover, stress-associated genes that have not yet been identified might contribute to the diverse survival of bifidobacteria upon oxidative stress, together with metabolic and physiological characteristics, as discussed in section 4.3 and 4.4.

Some genes involved in the acid stress response in bifidobacteria, including polyphosphate kinase, glutamate-cysteine ligase and aminopeptidase P, were found to be highly conserved in the genus (Figure 14) (**Paper V**). In contrast, other acid stress-associated genes were present only in a subset of species. Oxalyl-CoA decarboxylase and formyl-CoA transferase homologs were only detected in the *B. pseudolongum* group, as well as in *B. pseudocatenulatum* and *B. dentium* strains. The species diversity associated with the prevalence of genes linked to acid stress may be a consequence of adaptation to specific natural environments.

The BSH-encoding gene was the only bile stress-associated gene subjected to genome analysis (Figure 14) (**Paper V**). The majority (94%) of analyzed *Bifidobacterium* strains were found to possess BSH homologs. Their occurrence in isolates of human and mammalian origin appears to be associated with the presence of bile salt in their natural environment. For example, isolates from the digestive tract of lemurs were found to lack a BSH homolog. Lemurs do not produce glycine-conjugated bile salts (Kirilenko et al., 2019), which are the preferred substrate of bifidobacterial BSH (Jarocki et al., 2014; Kim et al., 2004; Tanaka et al., 2000) and are more toxic than taurine-conjugated bile salts (Grill et al., 2000; Noriega et al., 2006).

The presence of genes encoding putative transcriptional regulators of stress responses such as WhiB-like family proteins (Geiman et al., 2006), varied across *Bifidobacterium* strains (Figure 14) (**Paper V**). This finding supports the assumption that stress-associated genes are most likely differentially regulated in *Bifidobacterium* strains, implying different levels of their protein products and diverse stress tolerance.

Comparison of the stress-associated gene profiles of BB-12 and BB-46 revealed that all but five candidates were shared between the two strains. Stress-associated genes that were found to be only present in either BB-12 or BB-46 are listed in Table 6. The absence of Hsp20, L-aspartate oxidase, and WhiB2 in BB-12, as well as that of oxalyl-CoA decarboxylase and formyl-CoA transferase in BB-46, was found to be a species-specific characteristic. As mentioned above, low sequence similarity plus different regulation of the shared stress-associated genes might contribute to the different stress tolerance exhibited by BB-12 and BB-46.

Table 6: Stress-associated genes that are not shared between BB-12 and BB-46.

Protein name	Function	Absent in
Hsp20	Small heat shock protein	BB-12
L-aspartate oxidase	Involved in *de novo* NAD^+ biosynthesis; might contribute to H_2O_2 formation	BB-12
WhiB2	Putative transcriptional regulator	BB-12
Oxalyl-CoA decarboxylase	Key enzyme of oxalate degradation	BB-46
Formyl-CoA transferase	Key enzyme of oxalate degradation	BB-46

Overall, genome analysis revealed potential links between stress tolerance and the stress response gene profile of various strains (**Paper V**). The stress response gene profiles of the 171 tested *Bifidobacterium* strains can guide future studies and identified additional key stress-associated genes that require further analysis. To improve genotype-phenotype correlations, increased phenotypic data on individual strains is required.

4.6 Summary

Systems-level characterization of the metabolism and physiology of BB-12 and BB-46 has provided new understanding of the stress physiology of these two strains. Genetic, metabolic, and physiological differences between BB-12 and BB-46 that have been identified in this work, their putative implications on cell growth behavior, as well as their robustness and stability are presented in Table 7.

Table 7: Identified genetic, metabolic, and physiological differences between BB-12 and BB-46 and putative implications on growth, robustness, and stability. BCP: bacterioferritin comigratory protein, Dps: DNA-binding protein from starved cells, ROS: reactive oxygen species, SFA: saturated fatty acids, UFA: unsaturated fatty acids.

	BB-12	**BB-46**	**(Putative) Implications on growth, robustness, and stability**	**Section**
Requirement for vitamins	Requirement for nicotinic acid, folate and pantethine	Requirement for menaquinone-4 and pantethine	Growth medium must fulfill nutrient requirements to ensure reproducible growth and high biomass yields Exposure to nutrient limitation may affect robustness and stability	3.4
Carbohydrate profile	Use of 9 out of 13 tested carbohydrates	Use of 12 out of 13 tested carbohydrates	Higher nutritional flexibility of BB-46 may contribute to survival in the gastrointestinal tract	3.4
Fermentation end products	Main fermentation end products: acetate and lactate	Depend on medium composition; in medium containing (i) only free amino acids: acetate and formate; (ii) a complex nitrogen source: acetate, lactate	Ratio of produced metabolites affects the ATP yield as well as redox balancing in bifidobacteria	3.5, 4.4
Effect of storage conditions on robustness and stability (pH: 4.5, 5.5, 6.5; temperature: 2–4°C, 8–10°C)	Minor effect of storage pH and storage temperature	Considerable effect of storage pH and storage temperature; preferred storage conditions: pH 6.5 and 2–4°C	Selection of storage conditions is crucial for good stability of BB-46 during storage	4.2
Effect of harvesting time on robustness and stability	Minor effect of harvesting time	Higher robustness and stability when harvested in stationary as opposed to exponential phase	Choice of harvesting time is crucial for some, but not all *Bifidobacterium* strains	4.2, 4.3
Lack of stress-associated genes	Hsp20, L-aspartate oxidase and WhiB2	Genes for oxalate detoxification	Oxalate degradation may contribute to acid tolerance and probiotic effect of BB-12 in gastrointestinal tract	4.3, 4.5

Table 7: Continued.

	BB-12	BB-46	(Putative) Implications on growth, robustness, and stability	Section
Expression of stress-associated genes	Higher expression of chaperones, oxidative stress-associated genes (e.g., Dps and BCP), and proton-translocating F_1F_0 ATPase in stationary phase	Increased expression of genes associated with DNA repair in stationary phase	BB-12: Enhanced maintenance of protein quality BB-12: Enhanced detoxification of ROS and protection of macromolecules against oxidative damage BB-12: Enhanced acid tolerance BB-46: Improved maintenance of DNA integrity is linked to improved robustness and stability in stationary phase	**4.3**
Amino acid consumption and secretion	Strong consumption of L-methionine	Almost no consumption of L-methionine	L-methionine consumption in BB-12 may fuel activity of methionine-sulfoxide reductase under aerobic conditions, thereby contributing to ROS-scavenging, maintenance of protein homeostasis, and supply of free amino acids	4.3
Cell membrane fatty acid profile	Dominant fatty acids: palmitic acid and oleic acid; cyclic fatty acid content ≤ 2.4%; UFA/SFA ratio ≤ 1.2, lower in the absence of Tween® 80	Dominant fatty acids: oleic acid > palmitic acid; cyclic fatty acid content: 8.1–12.4%. UFA/SFA ratio: 2.9–8.0; natural selection of fatty acid synthase variant in the absence of Tween® 80, showing decreased UFA/SFA ratio and improved survival during storage	BB-46 requires exogenous fatty acid source for growth. Cell membrane fatty acid profile affects robustness and stability of strains. A UFA/SFA ratio around or below 1 was found to improve survival during aerobic short-term refrigerated storage	4.3, 4.4
Polyphosphate accumulation	Yes	Yes	Polyphosphate accumulation may contribute to acid tolerance	4.3
Hydrophobicity	Hydrophobic cell surface	Hydrophilic cell surface	Sedimentation of BB-12 cells during storage may minimize exposure to environmental stressors, such as oxygen and acid, compared to BB-46	4.3, 4.4
Cell size	Larger size than BB-46; increased cell size in complex MRS medium, coinciding with decreased survival during storage	Smaller in size than BB-12	A larger cell size of BB-12 when cultivated in complex MRS medium may increase stress susceptibility due to a greater surface area being exposed to stressors	4.4

5 Conclusions

In this thesis, the metabolism and physiology of the industrially and clinically relevant *Bifidobacterium* strains BB-12 and BB-46 were studied following a systems biology approach that combined metabolic modeling, omics technologies, as well as classical metabolic and physiological analyses. The aim was to identify key factors that impacted on growth, viability, and stability, thereby paving the way for knowledge-driven development of efficient industrial production of highly robust and stable probiotic bifidobacteria.

To reach this aim, the thesis was organized around seven secondary goals.

1. **To reconstruct high-quality genome-scale metabolic models describing the metabolic capabilities of BB-12 and BB-46.**

Manually curated genome-scale metabolic models describing the metabolic capabilities of BB-12 and BB-46 were reconstructed. In contrast to previously developed metabolic models of *Bifidobacterium* strains, these included a *Bifidobacterium*-specific biomass objective function, were manually curated, and were qualitatively and quantitatively validated against experimental data. Application of the metabolic models in a constraint-based modeling framework fostered new hypotheses describing the metabolic landscape of bifidobacteria, such as the pathways contributing to succinate production (**Paper I**).

2. **To develop a chemically defined medium supporting reproducible growth of BB-12 and BB-46 using a model- and data-driven approach.**

The high-quality models were shown to be a suitable tool to guide the design of a chemically defined medium. In contrast to complex media, the new formulation enabled the assessment of individual medium components on growth of BB-12 and BB-46. Knowledge on the nutritional requirements of probiotic bifidobacteria is essential to develop and optimize industrial cultivation processes (**Paper I**).

3. **To identify the nutritional requirements of BB-12 and BB-46.**

The high-quality models and the chemically defined medium were proven to be of high value to identify the nutritional requirements of BB-12 and BB-46. While the two strains were found to require different sets of vitamins, both were found to only require L-cysteine or L-methionine as sole amino acid and sulfur source. In addition, the capability to ferment different carbohydrates varied between the strains (**Paper I**).

4. **To review existing literature on the stress response in bifidobacteria.**

Summarizing the literature on the stress response in bifidobacteria highlighted a dearth of studies on the mechanisms underlying the varied robustness of *Bifidobacterium* strains or the molecular determinants for strain stability. Identified knowledge gaps included limited information on the response of bifidobacteria to osmotic and cold stress, as well as on the functionality of stress-associated genes (**Paper II**).

5. **To identify key differences in the metabolism and physiology of BB-12 and BB-46.**

A comparative analysis of BB-12 and BB-46 in pH-controlled, lab-scale batch cultivations revealed considerable differences in the metabolism and physiology of the two strains (**Paper III**). In line with previous studies, the robustness and stability of BB-12 was superior

to that of BB-46. BB-12 and BB-46 had different maximum specific growth rates, substrate utilization profiles, and metabolite production. A higher uptake rate of L-methionine in exponential phase and an upregulation of L-methionine biosynthesis in stationary phase may be attributed to enhanced activity of ROS-detoxifying (peptide)-L-methionine-(R/S) sulfoxide reductase in BB-12, and thus explain improved survival under aerobic conditions compared to BB-46. Moreover, BB-12 displayed a constantly higher expression of several stress-associated genes, suggesting that its strong robustness may derive from an intrinsic preparedness to defend itself against the detrimental effect of environmental stressors.

BB-46 showed better survival during short-term storage when harvested in stationary compared to exponential phase. This growth phase-dependent phenotype might be due to the upregulation of genes involved in DNA repair during stationary phase.

Further differences between BB-12 and BB-46 concerned the composition of the cell envelope, which may also explain the divergent stress physiology of the strains. While BB-12 had a hydrophobic cell surface, BB-46 had a hydrophilic one. Moreover, compared to BB-46, BB-12 showed a lower ratio of unsaturated to saturated fatty acids in its cell membrane.

Taken together, several cellular characteristics appear to contribute synergistically to the superior robustness and stability of BB-12 relative to BB-46.

6. To investigate the influence of medium composition on growth, robustness and stability of BB-12 and BB-46.

Testing different medium compositions for the propagation of BB-12 and BB-46 highlighted the strong impact of nutrient supply on growth, but also on robustness and stability of BB-12 and BB-46 (**Paper IV**).

Although robustness and stability of both strains were affected by the medium composition, BB-12 exhibited better survival during short-term storage across all tested media. In contrast, robustness and stability of BB-46 were particularly poor after cultivation in complex medium and in semi-chemically defined medium with casein hydrolysate. This finding suggests that a complex nitrogen source lowers stress tolerance in BB-46 and, consequently, impairs viability during storage.

BB-46 failed to grow in the absence of an exogenous fatty acid source. Its inoculation in medium free from fatty acids resulted in the natural selection of a variant with a single nucleotide polymorphism in the gene encoding a type I fatty acid synthase. The mutant showed enhanced robustness and stability compared to its parental strain, as well as a similar fatty acid profile as BB-12, with a ratio of unsaturated to saturated fatty acids around one. In turn, BB-12 and BB-46 showed particularly low robustness and stability in MRS medium, along with a higher ratio of unsaturated to saturated fatty acids in their cell membrane. These results suggest that a ratio of unsaturated to saturated fatty acids below or around one improves robustness and stability in bifidobacteria.

When cultivated in complex medium, BB-12 showed considerably lower robustness and stability than in all other media. This result may be linked to the larger cell size of BB-12, which exposed a greater surface area to environmental stressors.

BB-12 showed greater cell surface hydrophobicity compared to BB-46 across all media. However, higher hydrophobicity alone does not seem to be sufficient to ensure high robustness and stability in bifidobacteria (**Paper IV**).

7. **To examine the genetic diversity of bifidobacteria, including BB-12 and BB-46, in terms of the prevalence of stress-associated genes**.

A genomic study on the presence and absence of 73 stress-associated genes in 171 genome-sequenced *Bifidobacterium* strains provided an overview of species diversity with respect to the prevalence of such genes (**Paper V**). Furthermore, the analysis allowed postulating putative phenotype-genotype correlations. For example, the absence of several oxidative stress-associated genes in *B. angulatum* and *B. adolescentis* strains may explain their exceptionally high O_2-sensitivity.

Overall, the results of the thesis have delivered new insights into the genetic, metabolic, and physiological characteristics of bifidobacteria, as well as differences between them, which will facilitate the rational improvement of their industrial production.

6 Future perspectives – towards rational optimization of industrial production of probiotics

Bifidobacteria are widely used as probiotics. The steadily increasing public awareness of the benefits offered by probiotics (Chin-Lee et al., 2014), has led to a rapidly growing market for products containing these microorganisms. In addition, there is a mere need for sustainable management of resources to stop the destructive exploitation of our planet. Therefore, effective, and resource-efficient production of probiotics is of increasing importance.

The optimization of industrial processes for the production of probiotic bifidobacteria has been largely based on traditional one-factor-at-a-time changes and statistical approaches, which are time-consuming and often fail to identify the molecular basis underlying a desirable phenotype. In contrast, rational optimization approaches rely on understanding the molecular basis of metabolic and physiological characteristics of bifidobacteria including their varying stress response, and aim for knowledge-based decision making when selecting new probiotic candidate strains for commercialization. In the course of this thesis, systems-level analysis of *Bifidobacterium* strains has resulted in the development of valuable tools and brought new insights, which can direct the rational optimization of industrial production of probiotics.

The chemically defined medium developed in this thesis (**Paper I**) should be applied to identify nutrients which promote growth and cell viability of *Bifidobacterium* strains, and whose addition to industrial media may improve process efficiency. In addition, the chemically defined medium may be employed as a pharma-grade substrate to produce probiotic strains for biotherapeutic applications.

Moreover, the experimentally validated, high quality genome-scale metabolic models of BB-12 and BB-46 can serve as templates for the generation of models of other *Bifidobacterium* strains, thereby shortening the time needed for their curation (as suggested in **Paper I**). As demonstrated in this thesis, metabolic models of industrially and clinically relevant *Bifidobacterium* strain provide different opportunities for guiding the optimization of industrial production, by serving as a queryable knowledge base. For one, they could be used to identify the nutritional requirements of commercialized *Bifidobacterium* strains, which can guide the optimization of production media and will facilitate troubleshooting, e.g., when the production performance is affected by batch-to-batch variations in complex media compounds.

In the presented work, the metabolism and physiology of BB-12 and BB-46 were compared in batch-cultivations under seemingly non-stressed conditions to assess for constitutively active mechanisms that contributed to the different robustness and stability of the two strains (**Paper III, Paper IV)**. In future studies, the direct influence of stressors on metabolism and physiology should be investigated. For this purpose, BB-12 and BB-46 can be cultivated in chemically defined medium and their response to different levels of stressors can be studied by transcriptomics or proteomics. The defined medium conditions will facilitate the application of the metabolic models of BB-12 and BB-46 for the interpretation of the resulting responses. These experiments may increase our understanding of the molecular

basis of the exceptionally high microbial robustness displayed by BB-12 and direct knowledge-driven selection of novel stable and robust strains based on genetic and/or cellular characteristics.

Another application of the presented results involves the use of highly expressed stress-associated genes in BB-12 as biomarkers of stability and robustness in bifidobacteria, e.g., the bacterioferritin comigratory protein related to oxidative stress (**Paper III**). To this end, expression of the candidate gene can be assessed under various conditions in BB-12 and other strains, and its level can be mapped against the robustness and stability of the studied strains under the given conditions. Once validated, the expression of these biomarkers could be determined in *Bifidobacterium* strains directly after cultivation. Accordingly, different cultivation conditions could be evaluated in real-time for their ability to deliver robust and stable strains, thereby avoiding the need for time-consuming robustness and long-term stability tests. Furthermore, it would be interesting to assess if short treatments with sub-lethal stress after cultivation can induce expression profiles similar to that of BB-12 also in other strains, thereby enhancing their survival during downstream processing, formulation, storage, and administration.

The development of genetic tools for bifidobacteria has achieved great progress in the recent years (Fukiya et al., 2018). The application of genetic tools will allow researchers to determine the contribution of individual (stress-associated) genes to robustness and stability via knock-out, overexpression or heterogeneous expression, and thereby facilitate also the validation of conclusions made in this thesis.

In particular, it was suggested that a high expression of a gene encoding (peptide)-L-methionine-(R/S) sulfoxide reductase, which coincided with a high L-methionine uptake and an upregulation of L-methionine synthesis in stationary phase, could contribute to the high robustness and stability of BB-12 (**Paper III**). To test this hypothesis, the effect on robustness and stability of BB-12 should be studied by (i) growing BB-12 in the absence of L-methionine and (ii) deleting the gene encoding (peptide)-L-methionine-(R/S) sulfoxide reductase. Moreover, the effect of increasing the L-methionine supply on robustness and stability of relevant strains should be assessed in industrial media. The importance of other highly expressed stress-associated genes in BB-12 should also be investigated using genetic engineering.

Genetic engineering studies can further contribute to the validation of genotype-phenotype correlations that were postulated in this thesis based on the genome-wide assessment of stress-associated genes in bifidobacteria (**Paper V**). The effect of knocking out the Dps gene in *B. dentium* could be investigated to assess its importance in O_2-tolerance. Another approach would be to evaluate how heterologous expression of missing oxidative stress-associated genes in *B. angulatum* and *B. adolescentis* impacts their O_2-tolerance. Comparing the O_2-tolerance of *B. adolescentis* strains that harbor different sets of oxidative stress-associated genes may contribute to a better understanding of these genes' function. Another requirement to allow for better understanding of the correlation between the presence of stress-associated genes and stress tolerance of *Bifidobacterium* strains is a systematic assessment of the impact of various stressors on growth and survival of a large group of genome-sequenced strains.

Across publishes studies, many different stress treatments have been used for assessing the robustness of probiotic bifidobacteria. The comparability and interpretation of data obtained across different studies could be improved by defining a standard condition for the evaluation of robustness and stability (stress-test) (as concluded in **Paper II**).

Overall, further research efforts are required to achieve an in-depth understanding of the stress response elicited in industrially and clinically relevant *Bifidobacterium* strain. In this thesis, knowledge gaps in the field have been filled, applying a systems-level characterization of *Bifidobacterium* strains. To find answers on the remaining open questions regarding the metabolism and physiology of bifidobacteria the combination of systems-level characterizations with classical molecular studies will be indispensable.

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