Survival of oxidative stress-adapted *Bifidobacterium* spp. in yoghurt

by

Ursula Louise Thomashoff

Submitted in fulfilment of the requirements
for the degree MSc Food Science

In the Faculty of Natural & Agricultural Sciences
University of Pretoria
Pretoria

May, 2024

DECLARATION

I, **Ursula Louise Thomashoff**, declare that the thesis, which I hereby submit for the degree, MSc Food Science, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: .....

DATE: .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Prof. Elna M. Buys for her guidance and encouragement throughout this project. Her mentorship and the significant opportunities she has provided have greatly enhanced my skills in the Food Science industry.

I am also deeply grateful to Dr. Thulani Sibanda for his unwavering mentorship and dedication to our research. His substantial commitment of time and profound insights have been invaluable to this project. Thank you for guiding me with such generosity and steadfast support — it has truly made a significant impact on my work and growth as a researcher.

I extend my thanks to the staff of the Food Science Department for their knowledge, expertise, constructive criticism, and kindness.

I am thankful to the University of Pretoria and MilkSA for their financial support, without which this study would not have been possible.

To the nearly Dr. Tlaleo Marole, who has been both a tremendous mentor and a cherished friend, your guidance has enriched both my personal and academic life immeasurably.

Thanks to Mrs. S. September from the Department of Biochemistry, Microbiology, and Genetics for her assistance with flow cytometry, and Mrs. E. van Wilpe from the Laboratory for Microscopy and Microanalysis for her help with sample preparation and microscopy analysis.

I am profoundly grateful to my parents and sisters for their constant presence and steadfast support, guiding and uplifting me throughout my studies.

I thank Greg for his love, patience, and understanding, which have been my foundation throughout this journey.

To my grandparents, thank you for your constant support and pride in my achievements.

To Kirsten, Lexi, Lisah, and Yolanda, thank you for more than six years of friendship and support.

Special thanks to Chris and Inge for their help during crucial times.

Lastly, and most importantly, to my two dogs, Bella and Obi, whose presence provided tranquillity during challenging times and endless 'dopamine hugs', making this journey not just bearable but joyful.

ABSTRACT

Survival of oxidative stress-adapted *Bifidobacterium* spp. in yoghurt.

by

Ursula Louise Thomashoff

Supervisor: Prof. Elna M. Buys

Co-supervisor: Dr. Thulani Sibanda

Degree: MSc Food Science

Bifidobacterium species are prominent constituents of the gut microbiota of healthy humans whose presence in the gut is linked with several health benefits. The supplementation of *Bifidobacterium* spp. as a probiotic through foods such as yoghurt is considered to be an effective way of sustaining a healthy gut microbiome and preventing gut dysbiosis. However, the ability to maintain the viability of *Bifidobacterium* spp. above the recommended therapeutic dose during the production and storage of yoghurt remains challenging due to its susceptibility to oxidative stress. This study aimed to investigate the effect of oxidative stress adaptation treatments on the physiological responses of three *Bifidobacterium* species, *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis*. The study also sought to isolate stress-adapted *Bifidobacterium* spp. variants and subsequently investigated their survival and viability during yoghurt manufacturing and storage shelf-life. Cultures of *B. bifidum*, *B. breve* and *B. animalis* were subjected to a sublethal (0.4 mM) hydrogen peroxide (H_2O_2) treatment followed by exposure to lethal (1 mM) H_2O_2 treatments across three successive generations. Membrane integrity and intracellular oxidation states of the H_2O_2-treated cells were evaluated using flow cytometry (FC) and fluorescent staining with SYTO 9 (S9) coupled with propidium iodide (PI), and CellROX® Green (CRG), respectively. The H_2O_2 treatments improved membrane integrity in *B. breve* and *B. animalis*, but increased intracellular oxidation states in all three *Bifidobacterium* species. Furthermore, the H_2O_2-treated cells were subjected to a lethal H_2O_2 challenge

(30 min; 1 mM H_2O_2) before combined FC analysis of membrane integrity and intracellular oxidation states using CRG with PI. Results showed that the H_2O_2 treatment had no effect on *B. breve* while improving the membrane integrity retention of *B. bifidum*, indicating potential adaptive changes that mitigated oxidative damage. *B. animalis* had the most distinct response in maintaining membrane integrity in an oxidised intracellular state, potentially reflecting the species' intrinsic oxidative stress tolerance. The morphological and ultrastructural characteristics and stress responses of stress-adapted *Bifidobacterium* cells were examined using scanning and transmission electron microscopy (SEM and TEM). *B. bifidum* consistently expressed extracellular vesicles (EVs), affecting the cell surface texture and possibly indicating disrupted cell division and granule formation – features that were enhanced following the lethal H_2O_2 challenge. Further adaptations and responses observed in *Bifidobacterium* spp. included cellular elongation, compaction of intracellular components, thinning of its cell envelope and surface texturing. *B. breve* also underwent cytoplasmic compaction for protection, whereas prominent circumferential rings observed on *B. animalis* enhanced cell aggregation and stress resistance. Finally, the adapted *Bifidobacterium* spp. were evaluated for their viability during yoghurt fermentation and storage, and their storage was compared to that of unadapted cells over 28 days. Although the viability of *B. bifidum* and *B. breve* declined during yoghurt storage, the stress adaptation resulted in better survival for both species during fermentation, suggesting that the stress adaptation may not be sufficient to protect the species against the combined effects of oxidative and acid stress during yoghurt shelf-life. Consistent with its known intrinsic stress tolerance, *B. animalis* maintained stable viability counts during yoghurt fermentation and storage. Bacterial viability was also determined using a novel propidium monoazide-quantitative polymerase chain reaction (PMAxx-qPCR) method. Interestingly, this culture-independent technique showed that all three *Bifidobacterium* spp. remained above the probiotic minimum level (6 log CFU/g) throughout storage. The results suggested a significant loss of culturability for some *Bifidobacterium* species and the potential transition into a viable but non-culturable (VBNC) state. Thus, the PMAxx-qPCR method may be a feasible option for accurate probiotic viability quantification that can account for cells in a VBNC state. The study shows that exposing *B. bifidum*, *B. breve*, and *B. animalis* subspp. *animalis* to sublethal- and subsequent lethal H_2O_2 treatments result in variants that are less susceptible to ROS-induced damage. Furthermore, the study confirms that stress adaptation is a promising method to enhance the viability of *Bifidobacterium* spp. during yoghurt manufacturing and

storage, maintaining recommended probiotic levels throughout the product's shelf life. This enhanced survival, attributed to an active oxidative stress response induced by adaptation treatments, suggests that oxidative stress adaptation is a feasible method to improve the survivability and functional stability of some probiotic *Bifidobacterium* spp. in yoghurt. This approach not only supports the maintenance of the minimum recommended probiotic levels (6 log CFU/g viable cells) throughout production and storage but also potentially extends the probiotic shelf-life of the yoghurt.

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

RESEARCH ARTICLES:

Sibanda, T., Marole, T.A., **Thomashoff, U.L.**, Thantsha, M.S., Buys, E.M. 2024. *Bifidobacterium* species viability in dairy-based probiotic foods: challenges and innovative approaches for accurate viability determination and monitoring of probiotic functionality. *Frontiers in Microbiology*, 15.

CONFERENCE PROCEDINGS:

National:

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2022. 'Oxidative Stress Adaptation of *Bifidobacterium* spp.', SASDT Dairy Student Evening, University of Pretoria, Pretoria, 25 August, 2022 (Oral presentation)

Sibanda, T., Marole, T.A., **Thomashoff, U.L.**, Hobden, A., Buys, E.M. 2023 'MilkSA Dairy Project', SASDT Symposium, Fordoun, Kwazulu-natal Midlands Nottingham Rd, 17 - 18 April, 2023 (Oral presentation)

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2023 'Oxidative Stress Adaptation: Potential Improvement of *Bifidobacterium* spp. Viability in Probiotic Yoghurt', SASDT Symposium, Fordoun, Kwazulu-natal Midlands Nottingham Rd, 17 - 18 April, 2023 (Poster presentation)

Marole, T.A., **Thomashoff, U.L.**, Hobden, A., Sibanda, T., Buys, E.M. 2023 'The Probiotic Viability Puzzle', 25th Biennial SAAFoST Congress, Cape Town International Convention Centre, Cape Town, 28 – 30 August, 2023 (Oral presentation)

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2023 'Maximising *Bifidobacterium* spp. Viability in Probiotic Yoghurt Production through Stress Adaptation', 25th Biennial SAAFoST Congress, Cape Town International Convention Centre, Cape Town, 28 – 30 August, 2023 (Poster presentation)

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2022 'Enhancing the Survival of *Bifidobacterium* spp. in a Probiotic Yoghurt through Adaptation to Oxidative Stress', SASDT Dairy Student Evening, University of Pretoria, Pretoria, 17 August, 2023 (Oral presentation)

International:

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2022 'Oxidative Stress Adaptation of *Bifidobacterium* spp.', 27th International ICFMH Conference FoodMicro 2022, Megaron International Conference Center, Athens Greece, 28 - 31 August, 2022 (Poster presentation)

Thomashoff, U.L., Sibanda, T., Buys, E.M. 2022 'Oxidative Stress Adaptation of *Bifidobacterium* spp.', 7th IDF Symposium on Science and Technology of Fermented Milk, Virtual, 29 – 30 November, 2022 (Poster presentation)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D-PAGE two-dimensional polyacrylamide gel electrophoresis

AFM atomic force microscopy

AhpC alkyl hydroperoxide reductase subunit C

ANOVA analysis of variance

BCAA branched-chain amino acids

CFU Colony-Forming Units

CRG CellROX® Green

Ct cycle threshold

cspA cold shock protein

ddH_2O double-distilled water

dNTP deoxynucleoside triphosphate

Dps DNA-binding protein

EMP Embden-Meyerhof-Parnas

EPS exopolysaccharide

EV extracellular vesicle

FC flow cytometry

GABA γ-aminobutyrate

GIT gastro-intestinal tract

HemN oxygen-dependent coproporphyrinogen III oxidase

HMDS hexamethyldisilazane

LAB lactic acid bacteria

Ldh2 lactate dehydrogenase

LDR linear dynamic range

LOQ limit of quantification

MALDI-ToF matrix-assisted laser desorption/ionisation time-of-flight

MIC minimum inhibitory concentration

MPD maximum population density

mRNA messenger RNA

MRS de Man, Rogosa and Sharpe (De Man et al., 1960)

MRS-C de Man, Rogosa and Sharpe media (MRS) (De Man et al., 1960) supplemented with L-cysteine (0.5 g/L)

NTC no-template control

OD optical density

ORP oxidation-reduction potential

PBS peptone buffered saline

PCA principal component analysis

PI propidium iodide

PMAxx propidium monoazide

PMAxx-qPCR propidium monoazide-quantitative polymerase chain reaction

PQC protein quality control

qPCR quantitative polymerase chain reaction

rRNA ribosomal RNA

ROS reactive oxygen species

RT-qPCR Real time qPCR

SCFA short-chain fatty acids

SEM scanning electron microscopy

SNP single nucleotide polymorphism

SOD superoxide dismutase

SPC standard plate counts

SPSS Statistical Package for the Social Sciences

S9 SYTO 9

TA titratable acidity

TEM transmission electron microscopy

tRNA transfer RNA

TrxR thioredoxin reductase

VBNC viable but non-culturable

VPI viability proportion index

CHAPTER 1

Problem statement

The human gut microbiota comprises a symbiotic community of bacterial and archaeal species and genera that reside in the gastro-intestinal tract (GIT) of healthy humans (Derrien et al., 2022). This microflora contributes several beneficial functions to the human host, including protection against gastrointestinal infections and non-communicable diseases such as gastric cancer and diabetes, enhancing host immunity, and improving digestion and nutrient absorption (Alessandri et al., 2021; Nyanzi et al., 2021; Derrien et al., 2022). *Bifidobacterium* species are a prominent constituent of the gut microbiota of healthy humans and contribute to various biological functions. These include enhancing intestinal function, reducing cholesterol levels, synthesis of B vitamins, and prevention of infectious diarrhoea and colon cancer (Nyanzi et al., 2021). However, factors such as age and diet can disrupt the natural balance of the gut microbiota, leading to dysbiosis (Arboleya et al., 2016; Shi, 2019). In particular, a decline in the relative proportion of *Bifidobacterium* spp. in the human GIT is known to occur with age (Arboleya et al., 2016). In infants and children, *Bifidobacterium* spp. constitute up to 90% of the gut microbiota, but this proportion declines to 10 - 40% in adults (Arboleya et al., 2016; Yang et al., 2021). This reduction has been linked to numerous adverse health outcomes that include gastrointestinal diseases, a weakened epithelial barrier, reduced immune responses, and overall gut discomfort (Hamilton-Miller, 2004).

The ability to supplement the bifidobacterial content of the human gut through probiotic foods is considered to be an effective way of restoring and sustaining a healthy gut microbiome (Sarkar, 2019). Among many foods that can serve as probiotic carriers, yoghurt is considered to be an optimal vehicle for probiotics due to its widespread popularity and distinctive matrix with a buffering capacity (Lourens-Hattingh and Viljoen, 2001; Zhang et al., 2023*a*). The probiotic microorganisms incorporated into foods for their beneficial health effects are required to maintain viability in the food product at or above a therapeutic minimum level of 6 log Colony-Forming Units (CFU)/g at the point of consumption (FAO/WHO, 2003; Nyanzi et al., 2021). For *Bifidobacterium* spp., the ability to incorporate and maintain its viability in foods

is a significant challenge (Sibanda et al., 2024). Its viability during yoghurt manufacturing is affected by its limited susceptibility to various stress factors encountered during the yoghurt fermentation and storage shelf-life (Schöpping et al., 2022; Sibanda et al., 2024). These stressors include heat, cold, acid, osmotic, and oxidative stress (Schöpping et al., 2022). Among the stress factors, oxygen is the most prominent factor potentially limiting the viability of *Bifidobacterium* spp. in yoghurt. As anaerobic organisms, *Bifidobacterium* spp. are generally sensitive to oxidative stress. However, their susceptibility varies among species and strains (Oberg and Broadbent, 2016; Schöpping et al., 2022). Some species, such as *B. boum* and *B. thermophilum*, are known to be hyper-aerotolerant, while others, such as *B. longum* subsp. *infantis* and *B. adolescentis* are oxygen-hypersensitive (Meile et al., 1997; Kawasaki et al., 2018*a*). The variation in aerotolerance among species suggests that even though *Bifidobacterium* spp. are anaerobes that generally lack the primary antioxidant enzymes like catalase and superoxide dismutase, some species possess alternative enzyme systems for protection against oxidative stress (Schöpping et al., 2022).

Several approaches aimed at enhancing the survival of *Bifidobacterium* spp. during yoghurt processing have been explored, with varying levels of success (Sibanda et al., 2024). These include microencapsulation, the addition of stress protective agents, and oxygen removal methods such as electroreduction (Dave and Shah, 1997*a*; Bazinet et al., 2009; Frakolaki et al., 2021). Another promising method is stress adaptation, which involves the exposure of *Bifidobacterium* spp. to sublethal stress treatments across multiple generations to induce adaptive responses and develop stress-resistant variants (Mozzetti et al., 2010; Schöpping et al., 2022). Despite *Bifidobacterium* spp. generally lacking the primary antioxidant enzymes, studies have shown that some species possess oxidative stress-inducible oxidoreductases that can help the organisms cope in the presence of oxygen (Oberg et al., 2015; Schöpping et al., 2023). Notably, enzymes such as alkyl hydroperoxide reductase subunit C (AhpC) and thioredoxin reductase (TrxR) (Oberg et al., 2015; Schöpping et al., 2023), together with several chaperones and reparative proteins with protective activities, have also been identified in *Bifidobacterium* spp. (Zuo et al., 2018). These protective mechanisms have been documented across various species, including *B. pullorum, B. pseudolongum, B. boum, B. adolescentis, B. longum, B. asteroides*, *B. bifidum*, *B. animalis*, and *B. breve* (Zuo et al., 2014; Tanaka et al., 2018; Satoh et al., 2019; Schöpping et al., 2022). The aim of this study is to investigate the effect of oxidative stress adaptation treatments on the physiological responses of three *Bifidobacterium* spp., namely *B. bifidum*, *B.*

breve, and *B. animalis* subsp. *animalis*. Furthermore, this study aims to isolate stress-adapted *Bifidobacterium* spp. variants that exhibit enhanced oxidative stress tolerance. Subsequently, this study investigates the survival and viability of the adapted variants during yoghurt manufacturing and storage shelf-life. Overall, this study focuses on enhancing the industrial application of *Bifidobacterium* spp. in the production of probiotic yoghurt.

CHAPTER 2

Literature review

The primary objective of this literature review is to offer a comprehensive understanding of *Bifidobacterium* spp. and its susceptibility to various stress factors, with a particular emphasis on oxidative stress encountered during yoghurt processing. Furthermore, the review investigates the probiotic's ability to respond and adapt to oxidative stress. It delves into the mechanisms governing the physiological, morphological, and genetic responses of *Bifidobacterium* spp. to oxidative stress. This review explores how these responses can be leveraged through sublethal and lethal stress exposures to develop variants that are better equipped to withstand oxidative stress during yoghurt production.

2.1 THE BIOLOGY OF BIFIDOBACTERIA

Bifidobacterium spp. are Gram-positive, non-spore-forming, non-motile rods, that belong to the *Bifidobacteriaceae* family. Most species assigned to this genus are the dominant inhabitants of the human GIT (Fanning et al., 2012). They are heterofermentative bacteria that produce varying amounts of lactate and acetate, depending on the substrate (Klijn et al., 2005). Currently, the genus consists of more than 80 classified *Bifidobacterium* species (Alessandri et al., 2021). Many species of *Bifidobacterium* have been found to be applicable in diverse industrial sectors, including clinical and food-related domains. Bifidobacteria are commonly categorised as lactic acid bacteria (LAB) due to their capacity to generate lactic acid during fermentation. However, they are phylogenetically distinct from other LAB, such as *Lactobacillus*, *Lactococcus*, and *Streptococcus*, which belong to the phylum Bacillota (previously Firmicutes), whereas bifidobacteria belong to the phylum Actinomycetota (previously Actinobacteria) (Vlasova et al., 2016; Oren, 2024). Furthermore, they heavily depend on complex carbohydrates for their energy requirements.

In contrast to other bacterial species, bifidobacteria do not produce the enzymes needed for the preparatory phase of the Embden-Meyerhof-Parnas (EMP) pathway typically associated with bacterial carbohydrate metabolism (O'Callaghan and van Sinderen, 2016). Specifically, key enzymes phosphohexose isomerase, phosphofructokinase-1, and aldolase. Instead, bifidobacteria utilise the *bifid shunt*, which employs two phosphoketolase enzymes, fructose 6-phosphoketolase and xylulose 5-phosphate phosphoketolase, to metabolise hexoses and pentoses, respectively (Pokusaeva et al., 2011). This enzyme facilitates the enzymatic breakdown of hexose phosphate into erythrose 4-phosphate and acetyl phosphate. Through the catalytic action of transaldolase and transketolase, acting on the erythrose 4-phosphate and fructose 6-phosphate, the xylulose 5-phosphate and ribose 5-phosphate are formed (Pokusaeva et al., 2011).

The glyceraldehyde 3-phosphate and erythrose-4-phosphate produced by the bifid shunt are then processed through the pentose phosphate pathway and the payoff phase of the EMP pathway, involving enzymes such as glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase (Pokusaeva et al., 2011). Eventually, in the bifid shunt pathway, fermentation involving two glucose molecules results in three acetate and two lactate molecules, and a higher ATP yield of 5 ATP molecules compared to the 4 ATP molecules produced by the EMP pathway (Shah, 2011). Palframan et al. (2003) have shown that *Bifidobacterium* spp. can effectively metabolise complex carbohydrates through its bifid shunt, leading to a higher energy yield than the EMP pathway. Notably, the lactate-to-acetate ratio is modulated by various factors, including the type of carbohydrate, culture conditions, specific species or subspecies, and growth phase (Shah, 2011).

2.2 BIFIDOBACTERIA AS A PROBIOTIC

The attractiveness of *Bifidobacterium* spp. for health-promoting food products lies in their diverse probiotic functionalities. A considerable amount of evidence from both in vitro and in vivo studies has confirmed the probiotic properties of *Bifidobacterium* species (Konieczna et al., 2012; Groeger et al., 2013; Turroni et al., 2014; Din et al., 2020; Shang et al., 2020; Zhang et al., 2020; Hee and Wells, 2021; Álvarez-Mercado et al., 2022; Schiweck et al., 2022;

He et al., 2023). *Bifidobacterium* spp. play a crucial role in enhancing the host's immune system by stimulating both innate and adaptive defences. They protect against infectious diseases, help prevent non-communicable diseases, and improve the host's nutritional metabolism (Turroni et al., 2014; Zhang et al., 2020; Schiweck et al., 2022; He et al., 2023).

Bifidobacterium spp. enhance the host's immune system by stimulating immune cells in the intestinal mucosa to secrete cytokines. Cytokines are crucial for the proliferation and differentiation of immune cells, playing a vital role in strengthening immune responses. Dong et al. (2010) showed that *Bifidobacterium* spp. plays a role in the growth of T cells in the thymus through the enhancement of the maturation of regional dendritic cells and IL-12 expression.

Furthermore, *Bifidobacterium* spp. actively contribute to the metabolism of dietary fibres such as arabinoxylans, pectin, and inulin, as well as human-derived heteroglycans, such as mucin and human milk oligosaccharides (Kelly et al., 2021; Li et al., 2023). Human digestive enzymes cannot metabolise these complex carbohydrates, leaving the task to the gut microbiota. The resulting products of this fermentation process, particularly short-chain fatty acidss (SCFAs), offer substantial health benefits. SCFAs help lower intestinal pH, enhance the bioavailability of essential minerals like calcium and magnesium, and inhibit the growth of pathogenic bacteria, thereby promoting overall health (Teitelbaum and Walker, 2002; Wong et al., 2006). Fanning et al. (2012) reported evidence that showed that *B. breve* UCC2003 can prevent pathogen colonisation and infection of the GIT through surface exopolysaccharide (EPS) production. Toure et al. (2003) identified several *Bifidobacterium* strains that produce heat-stable proteinaceous compounds exerting antimicrobial activity against *Listeria monocytogenes*. Similarly, the inhibitory activity of *B. longum* against *Candida* strains through the production of different metabolites was established by Inturri et al. (2019). These findings underscore the potential of bifidobacteria as a natural and effective means of combatting harmful pathogenic microorganisms.

2.3 YOGHURT AS A PROBIOTIC CARRIER

Yoghurt is a dairy product that results from the cooperative fermentation of milk with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* resulting in a smooth,

acid gel. It is widely recognised as an excellent carrier for probiotics, particularly *Bifidobacterium* spp., for several compelling reasons. Firstly, during the fermentation of milk, the gradual acidification of the environment allows *Bifidobacterium* spp. to acclimatise to the changing acidity (Lourens-Hattingh and Viljoen, 2001). This further protects the bacteria as it passes through the harsh acidic conditions of the stomach, enhancing its survival and effective delivery to the GIT (Egan et al., 2018; Matouskova et al., 2021).

Additionally, yoghurt offers a nutrient-rich environment comprising carbohydrates, proteins, lipids, minerals, and vitamins, which supports the viability and stability of probiotics (Lourens-Hattingh and Viljoen, 2001; Pokusaeva et al., 2011; Schöpping et al., 2021). The ability of *Bifidobacterium* spp. to produce acetic and lactic acid through the bifid shunt further supports its incorporation into yoghurt. Furthermore, some strains of *S. thermophilus* potentially acts as oxygen scavengers, contributing to the creation and maintenance of an anaerobic environment that benefits the growth and viability of *Bifidobacterium* spp. (Talwalkar and Kailasapathy, 2004*a*).

In South Africa, yoghurt is a popular dairy product, with a significant portion of the population incorporating it into their daily diets. According to the Consumer Education Project (CEP) report (2022), 48.3% of households consume yoghurt weekly, with consumption levels higher among affluent demographics. Given that yoghurt is already a staple in many diets, its use as a functional food to deliver beneficial probiotics could see excellent consumer acceptance, leveraging its role in regular consumption patterns to provide significant health benefits. Moreover, the yoghurt market is projected to increase annually by 6.95% (Statista, 2024). Therefore, yoghurt is an ideal vehicle for delivering *Bifidobacterium* spp. to the consumer, which can capitalise on the existing market penetration and positive health benefits associated with the consumption of yoghurt.

In the South African market, the options for probiotic yoghurt or yoghurt supplemented with *Bifidobacterium* spp. are currently limited. The available yoghurt products on the shelves contain either '*Bifidobacterium* (HN019) culture' (Woolworths, n.d.) or *Bifidobacterium lactis* BB-12 (Hansen, n.d.). However, the ability of the probiotic to maintain viability above the recommended therapeutic level for the duration of a 28-day shelf-life has yet to be published.

Preliminary experiments investigating the viability of *B. bifidum* ATCC® 11863™ during yoghurt fermentation and storage (12 d) were performed. *B. bifidum* viability significantly declined after 12 days of storage, with initial levels of 6.0 log CFU/g and a final level of 3.5 log CFU/g. Various factors affecting the probiotic viability were identified, including a decrease in pH, low temperatures, microbial interaction, and the incorporation of oxygen during processing. However, it was suggested that including oxygen during processing and storage and the acidic environment were the main factors contributing to the loss in bifidobacteria viability in the yoghurt (Thomashoff et al., 2021). The most challenging factor affecting the viability of *Bifidobacterium* spp. during yoghurt fermentation and storage, therefore, remains its sensitivity to oxygen, which will be explored in subsequent sections.

2.4 STRESS FACTORS AFFECTING VIABILITY DURING YOGHURT PROCESSING AND SHELF-LIFE

Note: The content of this section is adapted from our previously published article: ***Sibanda et al. (2024)****, Frontiers in Microbiology, 15, pp. 4-7.*

When it comes to the processing and shelf-life of probiotic yoghurt, various factors come into play that can impact the viability and functionality of *Bifidobacterium* species. This probiotic requires careful management of stress factors during the yoghurt production processes and shelf-life to survive, as illustrated in Figure 2.1. Stress factors typically associated with yoghurt products, such as heat, cold, acid, osmotic and oxidative stress, can significantly impact *Bifidobacterium* spp. survival and functionality in yoghurt. This section aims to explore the impact of the stress factors typically associated with the manufacturing and storage of yoghurt on the survival of *Bifidobacterium* spp. whilst delving into the ability of these bacteria to withstand and thrive under these varying stress conditions.

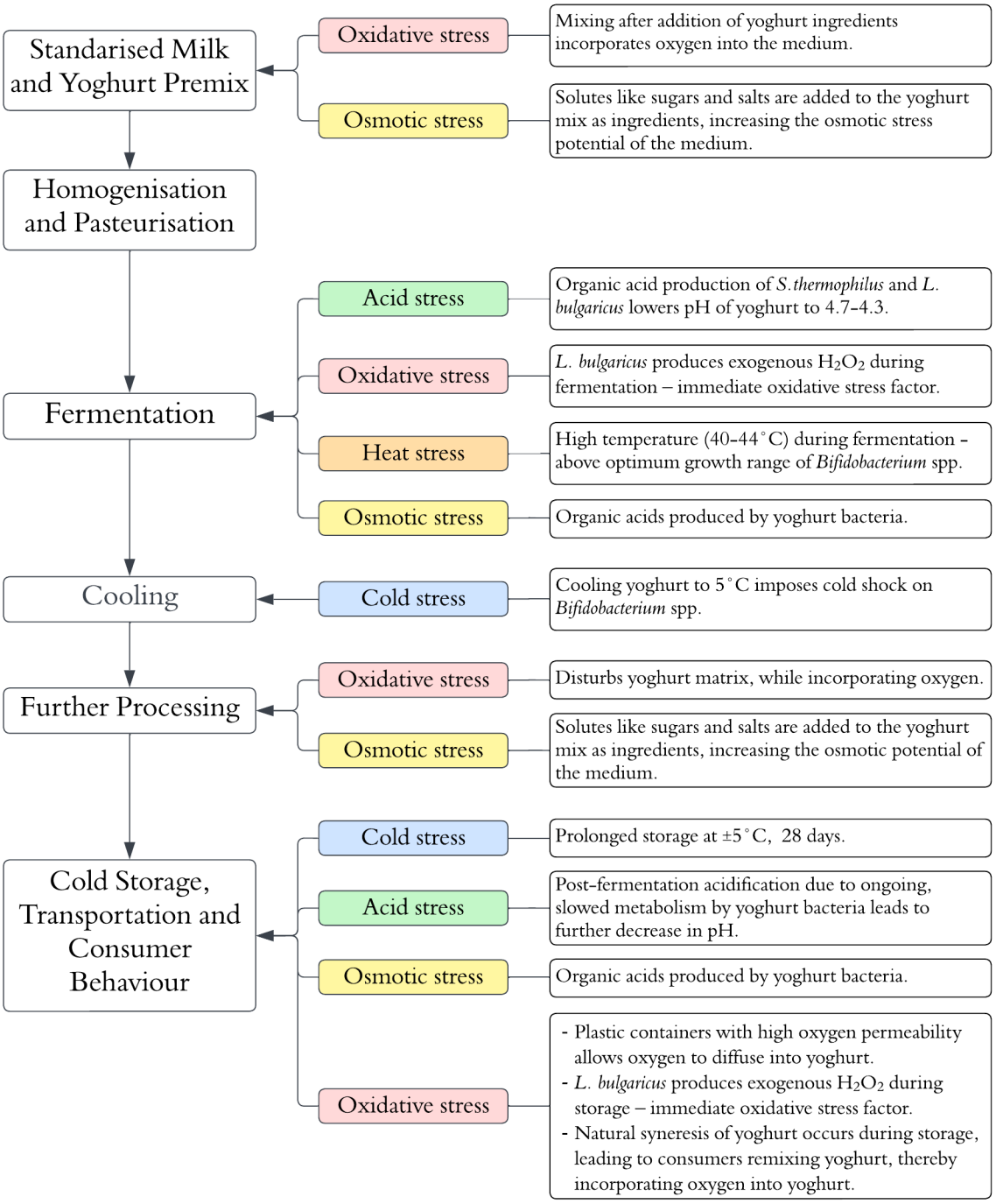


Figure 2.1 Stress factor integration points in the yoghurt production process and their respective impact on *Bifidobacterium* spp. viability. (Adapted from Figure 1 in **Sibanda et al. (2024)**)

2.4.1 Heat stress

Yoghurt production involves several thermal steps, including mix preparation, homogenisation, pasteurisation, inoculation, incubation, cooling, and further processing (Sfakianakis and Tzia, 2014). The fermentation step, occurring at 40 - 44 °C and pH 4.3 - 4.7, is crucial for defining the final product's characteristics. Incorporating *Bifidobacterium* spp. in yoghurt production is challenging due to their sensitivity to high temperatures, as they thrive at 37 - 41 °C (Gratteppanche and Lacroix, 2013).

Extensive research has explored the molecular mechanisms behind the heat stress response in *Bifidobacterium* spp., mainly focusing on various species' unique responses. Heat stress defence primarily involves the production of protective heat shock proteins and reduced metabolic activity. Notably, *B. longum* NCC2705 and *B. breve* UC2003 have been studied extensively in this context (Rezzonico et al., 2007).

High temperatures trigger the upregulation of genes related to heat shock proteins while inhibiting other critical biochemical pathways, potentially impacting cellular functions and survival (Rezzonico et al., 2007). *Bifidobacterium* spp. activates a protein quality control (PQC) system comprising transcriptional regulators and heat shock proteins to maintain protein homeostasis. The SOS system, activated by damaged DNA, further stimulates the PQC system in *Bifidobacterium* spp. (Zomer et al., 2009).

Heat shock proteins are induced not only by heat but also by other stressors (Sugimoto et al., 2008). *B. animalis* subsp. *lactis* BB-12 increases the production of the H_2O_2 detoxification enzyme, TrxR, after heat stress exposure, demonstrating the multifaceted role of heat shock proteins in *Bifidobacterium* spp. survival (Aakko et al., 2014). *Bifidobacterium* spp. possess various families of heat shock proteins, with chaperones playing a prominent role in the stress response of *B. breve* UC2003 (Ventura et al., 2006). The diversity of heat shock protein families underscores their vital function in responding to heat stress (Sugimoto et al., 2008). For a comprehensive analysis, refer to Schöpping et al. (2022) and Schöpping et al. (2022).

2.4.2 Cold stress

Once the pH of the yoghurt reaches 4.3 - 4.7, it is then chilled to approximately 5 °C to inhibit the growth and metabolic activities of the yoghurt bacteria (Sfakianakis and Tzia, 2014). In addition to the drop in temperature after fermentation, the yoghurt is stored at chilled temperatures for the duration of the shelf-life, typically 28 days. Cell counts of probiotic cultures usually decrease during cold storage (Beheshtipour et al., 2012), which is an undesirable effect of the processing conditions. Most bifidobacterial minimum growth range lies at approximately 25 - 28 °C, which is considered mesophilic (Grattepanche and Lacroix, 2013). While the impact of cold storage on the viability of numerous *Bifidobacterium* species has been extensively studied, the exact molecular mechanism behind the cold stress response remains to be investigated. However, a gene encoding for a cold shock protein (cspA), was identified by du Toit et al. (2013) within the genome of *B. breve* UC2003. The genetic sequence of the cspA gene shows a significant resemblance to cold shock proteins of other high-G+C bacteria (du Toit et al., 2013). Nevertheless, the genetic and metabolic foundations of cold stress responses in bifidobacteria await more comprehensive exploration.

2.4.3 Acid stress

Bifidobacterium spp. survival is greatly influenced by environmental conditions, particularly pH. Yoghurt, with its pH range of 4.7-4.3, poses an acidic challenge (Sfakianakis and Tzia, 2014). *Bifidobacterium* spp. typically thrive between pH 6.5-7.0 (Biavati and Mattarelli, 2006), making survival below pH 4.5 or above 8.0 challenging, except for select strains (Matsumoto et al., 2004; Mättö et al., 2004; Vernazza et al., 2006). Yoghurt fermentation, led by *S. thermophilus* and *L. bulgaricus*, produces organic acids, lowering the pH and potentially compromising *Bifidobacterium* spp. viability during storage (Deshwal et al., 2021). Maintaining intracellular pH homeostasis is critical for *Bifidobacterium* spp. under acidic conditions. They initiate an acid stress response involving proton extrusion and biomolecule repair, primarily driven by the proton-translocating F_1F_0-ATPase (Schöpping et al., 2022). This process demands substantial energy, with fermentable carbohydrates enhancing *Bifidobacterium* spp.'s ability to tolerate acidity (Waddington et al., 2010). However, the role of F_1F_0-ATPase varies among species.

Amino acid metabolism plays a role in acid tolerance. Strains like *B. longum* NCIMB 8809 increase branched-chain amino acids (BCAA) synthesis, aiding in intracellular pH regulation (Sánchez et al., 2007). Additionally, sulfur-containing amino acids, such as cysteine, contribute to acid stress response (Sánchez et al., 2007). Some *Bifidobacterium* strains can convert glutamine to aspartate, regulating pH (Eckel and Vogel, 2020). The glutamate decarboxylase pathway produces γ-aminobutyrate (GABA), consuming protons (Yunes et al., 2016; Duranti et al., 2020). Low pH conditions affect protein expression, including heat shock proteins and DNA repair molecules (Sánchez et al., 2007). Strain-specific responses vary, with some strains reducing protein synthesis for growth and enhancing proteins for survival (Jin et al., 2012). *Bifidobacterium* spp. adapt their cell membranes under acid stress, with changes in peptidoglycan content and fatty acid profiles (Jin et al., 2012; Wei et al., 2019). EPSs have a dual role, either enhancing or limiting acid resistance based on EPS production levels (Alp and Aslim, 2010; Jiang et al., 2016). These responses illustrate the diversity of mechanisms *Bifidobacterium* strains employ to survive acidic environments.

2.4.4 Osmotic stress

In yoghurt production, the addition of osmotically active solutes like sugars and salts, along with organic acids produced by yoghurt microbiota, imposes osmotic stress on *Bifidobacterium* species (Cui et al., 2016). Fluctuations in osmolarity can lead to water loss from cells, impacting intracellular osmotic pressure (Wesche et al., 2009). *Bifidobacterium* spp. responds by regulating intracellular osmolyte concentration (De Angelis and Gobbetti, 2004; Corcoran et al., 2008) and implementing protein DNA quality control and repair mechanisms (Ventura et al., 2005; Schöpping et al., 2022).

The response to osmotic changes in *Bifidobacterium* spp. is linked to maintaining cell wall integrity and turgor pressure. *B. longum* BBMN68 upregulates an efflux channel to reduce turgor pressure in response to bile exposure (An et al., 2014). *B. breve* UCC2003 activates a heat shock response, the extent of which depends on osmotic stress severity (Ventura et al., 2005). Variations in responses among *Bifidobacterium* strains highlight the need for further investigation into osmotic stress mechanisms and adaptations within this genus.

2.4.5 Oxidative stress

Throughout the production and storage of yoghurt, various mechanisms exist for oxygen to permeate the food matrix, thereby increasing the dissolved oxygen level. Such a phenomenon can have a detrimental impact on the viability of the probiotics. Notably, stirring, homogenising, mixing, further processing, and consumer food handling can all contribute to oxygen incorporation into the yoghurt. As depicted in Figure 2.1, it can be observed that oxidative stress is the most frequent stress factor *Bifidobacterium* spp. are subjected to during yoghurt production. Due to the absence of genes encoding catalase, NADH peroxidase, or superoxide dismutase (SOD) (Zuo et al., 2018), bifidobacteria are unable to detoxify H_2O_2 (Xiao et al., 2011) through systems typically associated with aerobic microorganisms (Zuo et al., 2014). They are classified as obligate anaerobes (Mozzetti et al., 2010). However, other mechanisms have been identified to alleviate the stress, which will be discussed in more detail in further sections (Talwalkar and Kailasapathy, 2004*a*; Zuo et al., 2018; Schöpping et al., 2022).

2.5 IN-DEPTH REVIEW OF OXIDATIVE STRESS

2.5.1 Sources of oxidative stress in yoghurt production

Although O_2 is not inherently toxic to *Bifidobacterium* spp., the formation of reactive species can lead to cellular damage and eventual loss of viability. This underscores the importance of understanding the mechanisms underlying oxidative stress in order to mitigate its adverse effects on these microorganisms. Bifidobacteria are classified as obligate anaerobes and are catalase-negative, which prevents them from metabolising and detoxifying oxygen through means typically associated with anaerobic bacteria (Shah, 2011). However, *B. indicum* and *B. asteroides* are exceptions to this rule (Schöpping et al., 2022; Schöpping et al., 2022). Both species possess SOD, an enzyme that converts superoxide radicals into H_2O_2 and O_2, thus protecting against oxidative stress. Additionally, *B. asteroides* possesses catalase, which further breaks down H_2O_2 into water and O_2, enabling it to detoxify reactive oxygen species (ROS) and survive conditions of oxidative stress (Schöpping et al., 2022; Schöpping et al., 2022).

While *S. thermophilus* has been reported to utilise oxygen during the fermentation of yoghurt, further processing steps have the potential to introduce additional oxygen (Talwalkar and Kailasapathy, 2004*a*). Furthermore, a study conducted by Dave and Shah (1997*b*) revealed that yoghurt stored in plastic containers exhibited a higher dissolved oxygen concentration after 35 days than in glass containers. While the use of glass containers for the preparation, processing and packaging of yoghurt is preferred due to their low oxygen permeability, they pose a risk of breakage, leading to increased transportation costs. As a result, Talwalkar et al. (2004) posited that glass containers are not suitable for yoghurt products as a vessel material.

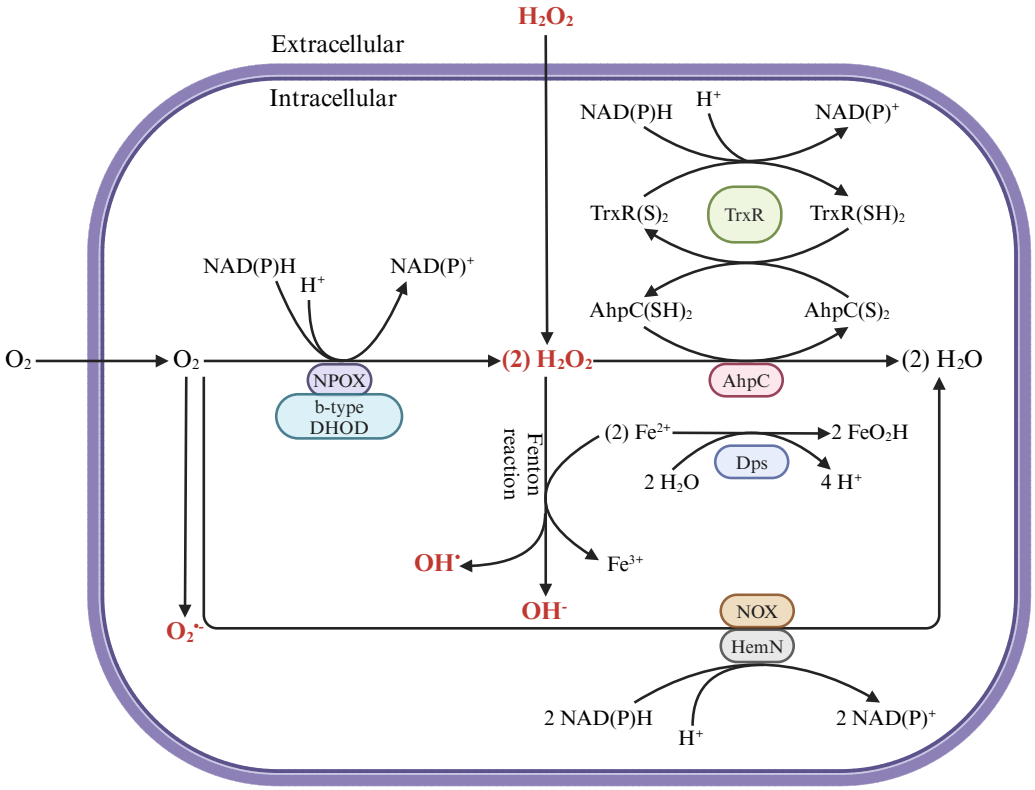


Figure 2.2 Formation of ROS and general response of *Bifidobacterium* spp. to oxidative stress. AhpC, alkyl hydroperoxide reductase C-subunit; DHOD, dihydroorotate dehydrogenase; Dps, DNA-binding ferritin-like protein; HemN, oxygen-independent coproporphyrinogen III oxidase; NOX, NAD(P)H oxidase (H_2O-forming); NPOX, NAD(P)H oxidase (H_2O_2-forming); TrxR, thioredoxin reductase. Adapted from Zuo et al. (2014) and Schöpping et al. (2022). Created with BioRender.com.

Due to the complex nature of the yoghurt matrix, various opportunities exist for the incorporation of molecular oxygen into the medium (Figure 2.1) and its subsequent interaction with compounds that results in the formation of toxic compounds (Figure 2.2). Previous studies have suggested that the growth of bacteria in yoghurt under aerobic conditions is mainly hindered by the breakdown of O_2 into H_2O_2 through the NADH oxidase reaction. The metabolic byproduct H_2O_2 has been proposed as the toxic agent that affects the viability of *Bifidobacterium* spp. in yoghurt (Talwalkar and Kailasapathy, 2004*b*; Kawasaki et al., 2009). Furthermore, during preliminary experiments, an antagonistic relationship between *B. bifidum* and the starter culture *L. bulgaricus* was identified (Thomashoff et al., 2021). *L. bulgaricus* is a potential source of exogenous H_2O_2 in yoghurt. Due to the yoghurt bacteria being incapable of entirely reducing oxygen, H_2O_2 and ROS accumulate as a result (Shah, 2000), leading to a disturbance in redox homeostasis.

2.5.2 Physiological effects of oxidative stress

When O_2 interacts with one, two, or three electrons, it results in the formation of ROS, typically through the Fenton reaction (Fenton, 1894). Another major process contributing to ROS formation in food products such as yoghurt, is the oxidation of organic compounds, particularly unsaturated fatty acids, which form hydroperoxides, which are inherently unstable (Citta et al., 2017). These hydroperoxides decompose through homolytic cleavage and produces damaging ROS (Juan et al., 2021; Citta et al., 2017). Some of the most prevalent ROS produced include hydroxyl radicals (HO·), superoxide radicals ($O_2{\cdot}^-$), lipid peroxy radicals (ROO·), and hydrogen peroxide (H_2O_2), as depicted in Figure 2.2 (Fenton, 1894; Hopkins and Neumann, 2019). The harmful effects of ROS arise from the oxidation of biomolecules, a process that can result in cellular death. In particular, bacterial cells are susceptible to ROS-induced damage, which manifests in the form of DNA and RNA damage, protein misfolding and aggregation, and lipid peroxidation (Imlay, 2008).

Fe^{2+} ions tend to bind selectively to certain regions within the DNA structure, often associating closely with specific DNA sequences (Fasnacht and Polacek, 2021). When ROS, such as H_2O_2 or O_2^-, is reduced by Fe^{2+}, the highly reactive resulting hydroxyl radicals (HO·) and hydroxide ions (HO^-) can directly react with, and damage the closely associated DNA (Figure 2.2). The most notable types of DNA damage include base modifications, single- and double-

strand breaks, and cross-linking (Fasnacht and Polacek, 2021). ROS can alter the bases within the DNA structure, with guanine being particularly susceptible (Candeias and Steenken, 1993). The oxidation of guanine leads to the formation of δ-oxo guanine, a modification known to mispair with adenine during DNA replication. This mispairing can result in GC-to-TA transversion mutations, which are potentially mutagenic and can alter genetic information crucial for the survival and function of the bacteria (Candeias and Steenken, 1993; Hsu et al., 2004).

In addition, ROS are capable of causing both single- and double-strand breaks in the DNA helix (Evans et al., 2004). These breaks can lead to severe genomic instability which, if not repaired, can result in cellular death. The repair processes themselves, while essential, can sometimes introduce further errors into the DNA sequence, affecting the cell's genetic integrity and subsequent biochemical processes (Chatterjee and Walker, 2017).

The cross-linking of DNA strands, as well as between DNA and proteins, as a result of ROS, can severely interfere with DNA replication and transcription, hindering essential cellular functions and resulting in dysfunctional cellular processes (Chatterjee and Walker, 2017; Torrecilla et al., 2024). Furthermore, oxidative stress can exert substantial damage on RNA, particularly ribosomal RNAs (rRNAs) and ribosomal proteins in *Bifidobacterium* spp., which play critical roles in protein synthesis (Seixas et al., 2022). ROS can induce modifications in rRNAs, which are integral components of the ribosome structure. These modifications can disrupt the precise assembly of ribosomes, as well as their functionality. Upon damage, the ability of the ribosomes to accurately translate the messenger RNA (mRNA) into functional proteins becomes compromised (Seixas et al., 2022). In turn, this leads to a reduction in the overall efficiency of protein synthesis, which is integral for cell growth and response to environmental stresses (Schöpping et al., 2022; Njenga et al., 2023).

In addition to rRNA damage, oxidative stress can also alter ribosomal proteins. These proteins are essential for the proper functioning and structural integrity of the ribosomes (Fasnacht and Polacek, 2021). Damage to ribosomes can lead to changes in their structure or function, potentially resulting in the synthesis of dysfunctional proteins, with severe implications for the normal biophysical properties of the proteins, affecting their folding and interaction with RNA (Shcherbik and Pestov, 2019).

Furthermore, when ribosomes are damaged, their ability to accurately match transfer RNA (tRNA) with mRNA codons diminishes, leading to errors in the translation process. Translation errors may lead to incorrect amino acids being included in proteins, resulting in non-functional or harmful proteins (Ou et al., 2019).

Damage to DNA, RNA, and ribosomes profoundly affects protein synthesis, but ROS can also directly damage the proteins themselves (Seixas et al., 2022). Damaged proteins may not correctly fold into their necessary three-dimensional structures, resulting in dysfunctional proteins that can aggregate within the cell and cause further cellular stress and impair normal cellular processes (Seixas et al., 2022). Several mechanisms to combat oxidative stress and restore redox homeostasis have been identified in *Bifidobacterium* species (Schöpping et al., 2022). However, many hypothetical compounds believed to be involved in the oxidative stress response of bifidobacteria have also been isolated and need further characterisation and classification (Schöpping et al., 2022).

2.5.3 Oxidative stress response in bifidobacteria

The defence and adaptation mechanisms of *Bifidobacterium* spp. against oxidative stress are complex and intricate, and the compounds expressed, mechanisms, and cellular components involved are species-specific and even strain-specific (Schöpping et al., 2022; Schöpping et al., 2022). *Bifidobacterium* spp. can be classified as either O_2-hypersensitive, O_2-sensitive, O_2-tolerant, or O_2-hypertolerant based on their inherent response and subsequent tolerance to oxidative stress (Kawasaki et al., 2018*a*). For example, *B. asteroides* and *B. indicum* are catalase-positive and, therefore, classified as O_2-tolerant, meaning that in the presence of oxygen, the bacteria can detoxify H_2O_2 to water (H_2O) (De Dea Lindner et al., 2008). Whereas *B. bifidum, B. breve* and *B. animalis* subsp. *animalis* are adversely affected by O_2 concentrations above 5%, thereby categorising them as O_2 sensitive species (Schöpping et al., 2022).

The primary oxidative stress response mechanisms of *Bifidobacterium* spp., as depicted in Figure 2.2, involve the reduction and detoxification of ROS, repair mechanisms for damaged cell components, changes in metabolic process expression, and alterations of the cell envelope (Zuo et al., 2018; Schöpping et al., 2022).

2.5.3.1 Reduction and detoxification of ROS

To sustain growth and confer therapeutic advantages to the host, bifidobacteria must employ mechanisms that mitigate oxidative stress in yoghurt. Under aerobic conditions, the enzymes NADH oxidase and an oxygen-dependent coproporphyrinogen III oxidase (HemN) are the key agents believed to be responsible for detoxifying molecular oxygen to H_2O_2 in bifidobacteria (Ruiz et al., 2012). However, *Bifidobacterium* spp. lack the genes typically associated with further conversion of H_2O_2 and other ROS into water (NADH peroxidase, catalase, and SOD), necessitating alternative detoxification strategies. The efficient scavenging of ROS by *Bifidobacterium* spp. is critical for their survival, as it helps them manage oxidative stress effectively. In *Bifidobacterium* spp., the detoxification of ROS primarily involves thioredoxin reductase (TrxR) and alkyl hydroperoxide reductase subunit C (AhpC) (Oberg et al., 2015; Satoh et al., 2019).

Alkyl hydroperoxide reductase, a flavin-containing disulfide reductase with an active disulfide centre, consists of two subunits, AhpC and AhpF, and is of the peroxiredoxin family of the thiol peroxidases (Poole et al., 2000). The AhpC component expresses peroxidase activity, while AhpF recycles oxidised AhpC; these are critical enzymes of the superoxide reductase-AhpC-rubredoxin pathway (Mukhopadhyay et al., 2007). Transcriptomic studies determined *B. longum* NCC2705 to possess a gene encoding for a protein similar to AhpC, yet *ahpF* was absent (Poole et al., 2000).

Under oxidative stress, TrxR catalyses the reduction of H_2O_2 to H_2O using NADH, thereby mitigating the harmful effects of H_2O_2 on cellular components (Figure 2.2). Due to the absence of AhpF, TrxR is redirected to recycle oxidised AhpC, ensuring continuous ROS scavenging and maintaining redox balance in *Bifidobacterium* species (Figure 2.2). Usually, TrxR is involved in the thioredoxin system, which is crucial for reducing the 2'-OH group of ribose in deoxynucleotide synthesis and maintaining cytoplasmic redox states for disulfide bond formation in proteins (Lu and Holmgren, 2014). Under oxidative stress, however, cells can utilise TrxR and AhpC to reduce H_2O_2 and other ROS, together with NADPH (Figure 2.2) (Zeller and Klug, 2006). Oberg et al. (2015) documented a significant upregulation of TrxR following H_2O_2 exposure in *B. longum*. Ultimately, TrxR is recycled by NADPH, continuing its role in the stress response, as illustrated in Figure 2.2. Numerous studies have confirmed the upregula-

tion of TrxR and AhpC in *Bifidobacterium* spp. when exposed to oxidative stress (O_2 or H_2O_2) (Xiao et al., 2011; Ruiz et al., 2012; Oberg et al., 2013; Zuo et al., 2014; Oberg et al., 2015; Tanaka et al., 2018; Zuo et al., 2018; Zhang et al., 2019).

2.5.3.2 Repair of damaged cell components

As previously mentioned, ROS can cause significant damage to intracellular components and result in the disruption of essential bioprocesses. For *Bifidobacterium* spp. to maintain its cellular integrity and functioning, it needs to efficiently and effectively repair any damage that the exposure to oxidative stress has caused to DNA, RNA and proteins.

Oberg et al. (2013) observed an upregulation of genes encoding proteins involved in the synthesis of deoxynucleoside triphosphate (dNTP) and DNA replication in *Bifidobacterium* spp. under oxidative stress, suggesting these molecular adjustments may facilitate the repair of oxidatively damaged DNA and RNA. Additionally, Xiao et al. (2011) reported that DNA-binding protein (Dps), a protein known for its role in DNA protection, was upregulated in *B. longum* BBMN68 following exposure to oxygen. Dps protects DNA by binding to it, effectively shielding the genetic material from ROS (Nair and Finkel, 2004). Moreover, Dps contributes to the detoxification of H_2O_2 by catalysing the oxidation of Fe^{2+} during the Fenton reaction, as illustrated in Figure 2.2. This dual function underscores the critical role of Dps during the oxidative stress response of *Bifidobacterium* species (Nair and Finkel, 2004).

In response to damage caused to proteins, *Bifidobacterium* spp. activate the PQC system to manage and mitigate the effects of oxidative stress. Xiao et al. (2011) observed an upregulation of the PQC regulon in *B. longum* BBMN68 when exposed to 3% oxygen. The PQC system encompasses molecular chaperones and proteases which facilitate the refolding of misfolded proteins or the degradation of irreversibly damaged proteins (De Dea Lindner et al., 2008). The activation of chaperones, such as DnaK, which was noted to be upregulated under oxidative stress conditions, helps in stabilising unfolded proteins, preventing their aggregation, and assisting in proper refolding (Zomer et al., 2009; Ventura et al., 2011).

Additionally, proteolytic systems like the Clp protease complex are crucial for degrading proteins that are beyond repair, thus preventing the accumulation of non-functional proteins

that could further stress the cell (De Dea Lindner et al., 2008; Zomer et al., 2009). By maintaining protein integrity and function through the PQC system, *Bifidobacterium* spp. enhance their resilience against oxidative stress, supporting their growth and viability in stressful environments.

2.5.3.3 Changes in metabolic process expression

A substantial amount of energy is necessary for *Bifidobacterium* spp. to effectively engage in these detoxification and repair mechanisms in response to oxidative stress. Consequently, oxidative stress can significantly alter the carbon uptake in *Bifidobacterium* spp., leading to notable changes in overall bacterial metabolism (Talwalkar and Kailasapathy, 2003; Ruiz et al., 2012). *Bifidobacterium* spp. utilise the *bifid shunt*, an alternative metabolic pathway that enables the fermentation of carbohydrates to optimise energy yield from sugars under anaerobic or microaerophilic conditions (Pokusaeva et al., 2011). Recent research on various *Bifidobacterium* strains has shown that these bacteria alter their metabolite production under oxidative stress conditions, favouring a higher proportion of acetate over lactate (Talwalkar and Kailasapathy, 2003; Ruiz et al., 2012). This shift is attributed to the competition for NADH between lactate dehydrogenase (Ldh2) and ROS detoxifying enzymes. As a result, carbon sources are redirected through the bifid shunt towards acetic acid production, generating more ATP than lactic acid production. This increased ATP yield enhances the energy available for the bacteria to cope with oxidative stress.

2.5.3.4 Cell envelope alterations

A critical stress response of *Bifidobacterium* spp. involves modifications of the cell shape, cell wall properties and fatty acid profile. Typically, species of bifidobacteria can be distinguished by its distinct Y, V or X *bifid* shape. However, recent studies indicated that the distinctive bifid shape of *B. adolescentis* is peculiar to this particular strain, regardless of cultivation conditions (Dhanashree et al., 2017). Whereas other strains of *Bifidobacterium* failed to retain their bifid morphology when grown in dissimilar environments (Dhanashree et al., 2017). Moreover, Ahn et al. (2001) documented that *Bifidobacterium* cells elongate and develop irregular cell surfaces in response to oxidative stress.

Recent research findings indicate a rise in short-chain and cyclopropane fatty acid levels under oxidative stress (Ruiz et al., 2007). As cyclopropane fatty acids are less susceptible to oxidation, this shift is suggested to assist *Bifidobacterium* spp. resilience to membrane damage caused by ROS (Oberg et al., 2011). Additionally, an increase in the levels of plasmalogens in the cell membrane of *B. animalis* subsp. *lactis* BL-04 was reported to contribute to an increased resistance to damage from intracellular oxidative stress (Oberg et al., 2011). This research strongly emphasises the importance of the ability of *Bifidobacterium* spp. to modulate its cell composition in its oxidative stress response.

It should be noted that the majority of studies regarding the ability of *Bifidobacterium* spp. to alter their morphology were conducted several decades ago (1900 - 2000) and may only partially apply to current research. Therefore, more recent investigations must be performed to elucidate the morphological plasticity of *Bifidobacterium* spp. further. The most recent findings by Zuo et al. (2018) indicate that exposure to low concentrations of H_2O_2 can lead to cell autoaggregation and increased cell surface hydrophobicity in the cells of *B. longum* BBMN68.

Figure 2.3 depicts the molecular responses of *Bifidobacterium* spp. to oxidative stress, which includes the activation of key detoxification enzymes, mechanisms for repairing DNA and protein damage induced by ROS, alterations in cellular metabolism, and adaptations in cell membrane composition.

2.6 ENHANCING TOLERANCE TO OXIDATIVE STRESS IN BIFIDOBACTERIA THROUGH STRESS ADAPTATION

The stress-adaptive response of *Bifidobacterium* spp. can be exploited to enhance or improve probiotic viability when incorporated into a harsh food environment, such as yoghurt (Mills et al., 2011). Numerous studies have explored how sublethal stress treatments impact the cellular strength of bifidobacteria while applying different stress factors, i.e. temperature, acid, solutes and oxygen (or H_2O_2) (Schmidt and Zink, 2000; Saarela et al., 2004; Mozzetti et al., 2010; Oberg et al., 2011; Tacconi et al., 2012). The activation of a stress response is connected to an inducible stress resistance, which leads to temporary physiological and metabolic adjustment to the stress condition.

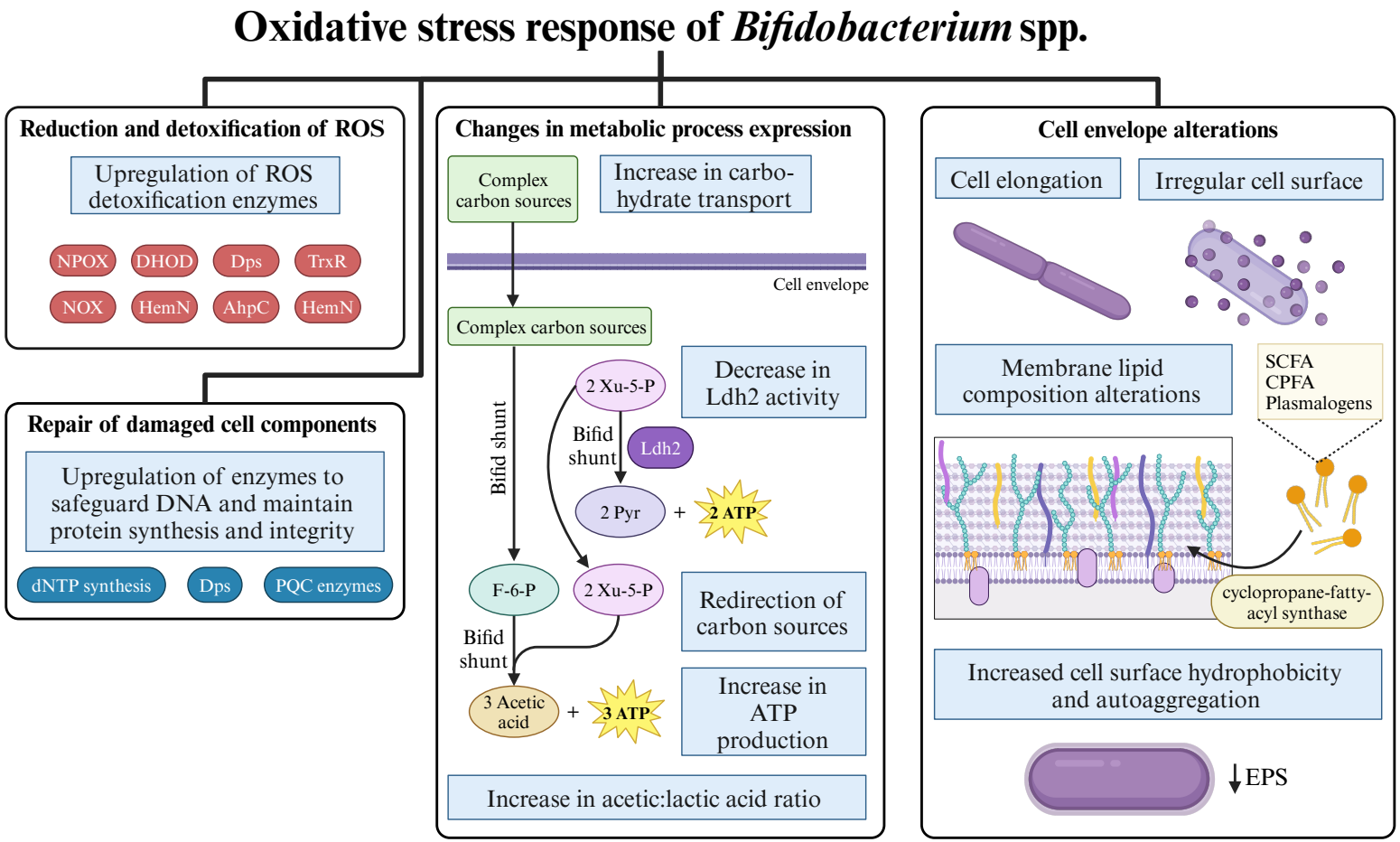


Figure 2.3 Molecular responses of *Bifidobacterium* spp. to oxidative stress. AhpC, alkyl hydroperoxide reductase C-subunit; CPFA, cyclopropane fatty acids; DHOD, dihydroorotate dehydrogenase; Dps, DNA-binding ferritin-like protein; EPS, exopolysaccharides; F-6-P, fructose 6-phosphate; HemN, oxygen-independent coproporphyrinogen III oxidase; Ldh2, lactose dehydrogenase; NOX, NAD(P)H oxidase (H_2O-forming); NPOX, NAD(P)H oxidase (H_2O_2-forming); PQC, protein quality control; Pyr, pyruvate; ROS, reactive oxygen species; SCFA, short-chain fatty acids; TrxR, thioredoxin reductase; Xu-5-P, xylulose 5-phosphate. Created with BioRender.com.

The physiological response of bacterial cells to stress factors effectively equips cells to handle larger amounts of stress in the future. It should be considered that temporary exposure to sublethal stress does not entail any genetic mutations and would consequently not result in any permanent alterations to the cell physiology, which may not be favourable in the yoghurt production industry (Schöpping et al., 2022).

Therefore, it is suggested to apply a combination of sublethal exposure and subsequent lethal stress exposure in combination, over several generations. This method would allow researchers to permanently induce the stress response without physically altering DNA, leading to stress-adapted strains of *Bifidobacterium*. A combination of sublethal exposure followed by lethal exposure will induce a physiological stress response that becomes a permanent physiological function, yielding *Bifidobacterium* variants with increased resistance to oxidative stress.

This method of stress adaptation will enable researchers to obtain variants of *Bifidobacterium* spp. that are genetically adapted to stress factors while avoiding complications involved with genetic engineering. However, it should be noted that the functionality of the probiotic should not be compromised in the process and should still be able to deliver its therapeutic effect in the host upon consumption.

Various researchers have successfully cultivated stress-adapted strains of *Bifidobacterium* spp. utilising heat, bile salts and acid stress (Noriega et al., 2004; Price et al., 2006; Sánchez et al., 2007; Berger et al., 2010; Yang et al., 2015; Jiang et al., 2016). In a study conducted by Mozzetti et al. (2010), the H_2O_2 stress-adapted bifidobacteria cells were isolated successfully using a continuous culture technique, essentially improving the bacteria's survival in the environmental stress by exposure to oxidative stress over an unlimited number of generations. Adapted strains were able to withstand higher levels of O_2 as compared to the wild-type strain (Mozzetti et al., 2010). Xiao et al. (2011) investigated the proteomic stress response of *B. longum* subsp. *longum* BBMN68 after exposure to 3% O_2 for 30 minutes and 60 minutes. The researchers observed an upregulation of genes encoding essential proteins involved in the protection or repair mechanisms of damaged cell components, such as AhpC, Dps, NrdA, and enolase. The expression of stress response proteins induced upon short and prolonged exposure to 3% O_2 distinctly differed from those expressed by other anaerobes while providing supporting information for applying *B. longum* in the food industry (Xiao et al., 2011).

Similarly, Zuo et al. (2018) exposed *B. longum* subsp. *longum* BBMN68 to 3% O_2 for 30 minutes and 60 minutes. However, they performed a global transcriptomic analysis of the induced oxygen response. It was identified that after 30 minutes of exposure to 3% O_2, the expression of 99 genes was downregulated, while 241 genes were upregulated. After 60 minutes of oxygen exposure, 218 genes were downregulated, and 217 were upregulated. The results obtained from the study confirmed that *B. longum* contains the genetic material to respond and adapt intrinsically to oxidative stress. The thioredoxin-thioredoxin reductase pathway was established as the primary defence system against ROS, and the expression of its enzymes experienced an approximate 2 log increase after 30 minutes of O_2 exposure (Zuo et al., 2018). These findings are crucial as they highlight the inherent oxidative stress response mechanisms in *B. longum*, supporting the potential for targeted genetic or environmental interventions to enhance the resilience of bifidobacteria during yoghurt production and shelf-life.

2.7 CONCLUDING REMARKS

Yoghurt is a popular dairy product with the potential to carry and deliver live and active probiotic cells to the consumer, conferring health benefits upon effective delivery to the distal GIT. Bifidobacteria are the main members of the human gut microbiome. Due to the health benefits associated with the organism, the inclusion of this probiotic in the formulation of a functional food product has gained interest. However, many species of bifidobacteria are oxygen-sensitive, meaning they do not grow well under aerobic conditions and, therefore, do not survive well during food processing. During yoghurt production, there are multiple opportunities whereby oxygen can be incorporated into the medium. The incorporation of oxygen during yoghurt production is a problem due to the formation of toxic and damaging ROS. To combat the effect of oxygen on the viability of bifidobacteria, researchers have investigated numerous methods, such as microencapsulation, cryoprotectant addition, modification of production processes and materials, and the inclusion of prebiotics.

A promising approach for enhancing the viability of *Bifidobacterium* spp. against oxidative stress involves subjecting the bacteria to sublethal stress, followed by successive cultivation under lethal stress settings in a stress adaptation treatment. This approach yields stress-adapted variants that can endure greater intensities of oxidative stress associated with the manufac-

turing of yoghurt. The oxidative stress response of certain strains of bifidobacteria has been characterised, including the genes involved in the expression of compounds responsible for detoxification, protection and repair mechanisms. Thus far, the only successful adaptation of *Bifidobacterium* spp. was performed using a continuous culturing technique by Xiao et al. (2011). The ability to produce an aerotolerant variant of *Bifidobacterium* spp. after exposure to sublethal oxidative stress, followed by successive cultivation under lethal oxidative stress, and to test the viability of the adapted isolates after exposure to previously lethal doses of oxidative stresses, remains unexamined.

Furthermore, adapted aerotolerant *Bifidobacterium* spp. has yet to be included in the processing and handling of yoghurt. The adaptation process aims to enhance the oxidative stress response of the probiotic, ultimately solving the problem of low *Bifidobacterium* spp. viability in a yoghurt environment.

CHAPTER 3

Hypotheses and objectives

3.1 HYPOTHESES

Hypothesis 1

Exposure of oxygen-sensitive strains of *Bifidobacterium* spp. to sub-lethal doses of hydrogen peroxide (H_2O_2) will induce aerotolerance, resulting in variants of *Bifidobacterium* spp., which are less susceptible to damage induced by reactive oxygen species (ROS). Due to the absence of genes encoding catalase, NADH peroxidase, or superoxide dismutase (SOD) (Schöpping et al., 2022), bifidobacteria are unable to detoxify H_2O_2 through systems typically associated with aerobic microorganisms (Oberg et al., 2015; Schöpping et al., 2022). They were previously classified as obligate anaerobes (Mozzetti et al., 2010). However, recent studies suggest a reclassification of bifidobacteria as either O_2-hypersensitive, -tolerant or -hypertolerant (Kawasaki et al., 2018*b*; Schöpping et al., 2022). This reclassification was suggested due to the identification of alternative enzymes, i.e. NADH oxidase, alkyl hydroperoxide reductase subunit C (AhpC), and thioredoxin reductase (TrxR), or homologues of such enzymes, found to be responsible for controlling the oxidative tolerance response in *Bifidobacterium* species (Oberg et al., 2015; Zuo et al., 2018; Schöpping et al., 2022). With exposure to oxygen, the following processes are induced in response to the oxidative stress: (1) the upregulated expression and activity of NADH oxidase, AhpC, and TrxR, or homologues of such enzymes, and (2) upregulated expression of proteins involved in the oxidative protection and repair of DNA, proteins and lipids, mainly chaperones and proteases (Xiao et al., 2011; Zuo et al., 2018; Schöpping et al., 2022, 2023). Under sublethal doses of H_2O_2, the upregulated expression of stress response enzymes and proteins whilst maintaining cell viability, can result in an adaptive stress response, which allows for *Bifidobacterium* spp. to develop oxidative stress tolerance (Mills et al., 2011; Zuo et al., 2018). Furthermore, by subsequently exposing the sublethal treated *Bifidobacterium* cells to lethal doses of H_2O_2 over successive generations and isolating survivors after each exposure, the previously induced stress response mechanisms can be permanently

integrated into the physiological, morphological and intracellular ultrastructural states of the cells (Mills et al., 2011; Oberg and Broadbent, 2016; Zuo et al., 2018; Schöpping et al., 2022). This sublethal- plus subsequent lethal H_2O_2 treatment results in the development of *Bifidobacterium* variants that are capable of surviving previously lethal doses of oxidative stress.

Hypothesis 2

Oxidative stress-adapted *Bifidobacterium* spp. variants will have higher viability and survival during yoghurt manufacturing and storage compared to unadapted cells and will maintain a viability level at or above the minimum recommended level for probiotics in yoghurt, i.e. 6 log CFU/g viable cells throughout the shelf-life. Oxidative stress-adapted *Bifidobacterium* spp. with an active oxidative stress response upon incorporation during yoghurt fermentation and storage have a sustained expression of NADH oxidase, alkyl hydroperoxide reductase subunit C (AhpC), and thioredoxin reductase (TrxR), or homologues of such enzymes (Talwalkar and Kailasapathy, 2003; Zuo et al., 2018; Schöpping et al., 2022), as well as several proteins involved in the oxidative protection and repair of DNA, proteins and lipids (Xiao et al., 2011; Schöpping et al., 2023). Following the addition to the yoghurt preparation before fermentation, adapted *Bifidobacterium* cells are exposed to molecular oxygen incorporated during prior processing. Subsequently, oxygen is reduced incompletely, forming damaging and toxic reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals, lipid peroxide radicals, and H_2O_2 (Averina et al., 2021). The enhanced oxidative stress response of stress-adapted *Bifidobacterium* spp. will effectively neutralise the detrimental effects of ROS on the bacterial cellular systems while detoxifying unreacted ROS, thereby preserving the intracellular redox balance and, subsequently, bacterial viability without compromising on yoghurt quality (Mohedano et al., 2022; Schöpping et al., 2022). Consequently, the stress-adapted *Bifidobacterium* spp. will exhibit increased resistance to oxidative stress during yoghurt fermentation and storage, maintaining a viability level at or above the minimum recommended level for probiotics in yoghurt, i.e. 6 log CFU/g viable cells throughout the shelf-life of the probiotic yoghurt (FAO/WHO, 2003; Schöpping et al., 2022).

3.2 OBJECTIVES

Objective 1

To characterise the physiological, morphological and ultrastructural adaptations of sublethal- and subsequent sublethal- plus lethal H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis* cells, with the aim of enhancing cellular adaptation strategies that could potentially improve their tolerance to future oxidative stress exposures.

Objective 2

To investigate the physiological and morphological characteristics, growth responses, and kinetics of the stress-adapted *Bifidobacterium* spp. variants when subjected to a lethal H_2O_2 challenge, with the aim of isolating variants of *Bifidobacterium* spp. with enhanced tolerance to lethal levels of oxidative stress.

Objective 3

To determine the effect of oxidative stress-adapted *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis*, on the physicochemical properties of yoghurt (i.e. pH, TA, ORP, syneresis) during refrigerated storage (28 days), with the aim of maintaining the appropriate acidity and a redox balance within the yoghurt throughout the storage period while ensuring an intact yoghurt gel.

Objective 4

To investigate the effect of oxidative stress adaptation of three probiotic *Bifidobacterium* species, i.e. *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis*, on their viability in yoghurt during fermentation and refrigerated storage, with the aim of sustaining the appropriate therapeutic dose (6 log CFU/g viable cells) of *Bifidobacterium* spp. cells throughout yoghurt fermentation and storage.

CHAPTER 4

Investigation of the physiological effects of stress adaptation on *Bifidobacterium* spp.

Bifidobacterium spp. are crucial to the healthy human gut microbiota and are linked to numerous health benefits. Incorporating *Bifidobacterium* spp. as a food probiotic can be a sustainable approach to countering gut dysbiosis. However, their viability in probiotic foods, such as yoghurt, is challenged by their sensitivity to oxidative stress during fermentation and storage. This study investigated the physiological, morphological and ultrastructural characteristics of H_2O_2-treated *Bifidobacterium* spp., to enhance their tolerance to oxidative stress. Cultures of *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis* were subjected to a sublethal (0.4 mM) H_2O_2 treatment followed by exposure to lethal (1 mM) H_2O_2 treatments for three successive generations. Flow cytometry assessed the membrane integrity of treated and untreated cells using SYTO 9 (S9) and propidium iodide (PI), whereas the oxidation states were assessed using CellROX® Green (CRG). Further, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses explored morphological and ultrastructural changes induced by the H_2O_2 treatments. The study showed enhanced membrane integrity of H_2O_2-treated *Bifidobacterium* spp., particularly in *B. breve* and *B. animalis*, with species-specific responses to oxidative stress. Elevated intracellular oxidation states were noted across all species, though some *B. bifidum* cells exhibited reduced oxidation, possibly due to activated detoxification mechanisms. SEM observations indicated the production of EVs with textured membrane surfaces following H_2O_2 treatment, suggesting structural adaptations to oxidative stress. Additionally, TEM analysis revealed cytoplasmic compaction in *B. breve*, aimed at protecting cellular components from oxidative damage. Future research will investigate the tolerance of the H_2O_2-treated *Bifidobacterium* cells to lethal oxidative stress, with the aim of optimising probiotic robustness during yoghurt production.

Keywords: *Bifidobacterium* spp., oxidative stress, stress adaptation, flow cytometry, membrane integrity, oxidation state, morphology

4.1 INTRODUCTION

Probiotics are defined as 'live microorganisms that confer a health benefit to the host when administered in sufficient quantities' (FAO/WHO, 2002; Hill et al., 2014). The recognition of the health benefits of probiotics has led to many probiotic species being incorporated into health-promoting foods. Among these health-promoting foods, yoghurt is considered to be an optimal vehicle for probiotics due to its widespread popularity and distinctive matrix with a buffering capacity (Lourens-Hattingh and Viljoen, 2001; Zhang et al., 2023*a*). *Bifidobacterium* species are an essential component of healthy human gut microbiota and are responsible for many health benefits, such as the prevention of gastrointestinal cancers, diarrhoeal diseases, necrotising enterocolitis and inflammatory bowel disease (Nyanzi et al., 2021). Hence, it has recently attracted a lot of focus as a potential probiotic for incorporation into fermented dairy products, particularly yoghurt (Zhang et al., 2023*b*).

As anaerobes, *Bifidobacterium* spp. are intrinsically sensitive to oxygen and its derived ROS. ROS are a group of molecules derived from redox reactions of molecular O_2 (Sies and Jones, 2020). The formation of ROS occurs when molecular oxygen accepts one, two or three electrons. The main ROS formed are hydroxyl radicals (HO·), superoxide radicals ($O_2{\cdot}^-$), lipid peroxide radicals (LOO·), and H_2O_2 (Hopkins and Neumann, 2019). ROS are toxic to bacterial cells. They cause damage to DNA, cause protein misfolding and aggregation, and lipid peroxidation (Imlay, 2008). During the production of yoghurt, various opportunities exist for oxygen to be incorporated into the food matrix, such as stirring, homogenisation, mixing and agitation (Sibanda et al., 2024). Furthermore, the exogenous production of H_2O_2 by adjacent yoghurt cultures can contribute to the loss of *Bifidobacterium* spp. viability (Meybodi et al., 2020). Therefore, challenges associated with the incorporation of *Bifidobacterium* spp. during yoghurt processing and the maintenance of appropriate therapeutic dosages in the product during storage remain a challenge, owing to the probiotic's susceptibility to the oxidative stress (i.e. ROS) encountered during the product's lifetime (Sibanda et al., 2024).

Stress adaptation refers to the process by which organisms adjust their physiological, metabolic, or genetic responses to cope with environmental stressors, thereby enhancing their survival and functionality under adverse conditions (Schöpping et al., 2022). The adaptation of *Bifidobacterium* spp. to oxidative stress can involve the activation of stress response pathways that lead

to changes in cell structure, function, and gene expression (Oberg et al., 2011; Schöpping et al., 2022). This adaptive response of *Bifidobacterium* spp. can be exploited to enhance or improve probiotic viability when incorporated into a harsh food environment (Mills et al., 2011). The stress adaptation process involves the pre-exposure of an organism to a sublethal dose of stress, which activates a stress response without compromising viability (Fiocco et al., 2020). This is subsequently followed by several repetitive generations of exposure to lethal doses of stress, isolating survivors after each exposure (Jiang et al., 2016). Using a similar approach, Mozzetti et al. (2010) successfully isolated an oxidative stress-adapted *Bifidobacterium* strain that exhibited moderate tolerance to oxidative stress. Therefore, the objective of this study was to characterise the physiological, morphological and ultrastructural adaptations of sublethal- and subsequent sublethal- plus lethal H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis* cells, with the aim of enhancing cellular adaptation strategies that could potentially enhance their tolerance to future oxidative stress exposures.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial cultures used in the study

B. bifidum ATCC® 11863™, *B. breve* ATCC® 15700™, and *B. animalis* subsp. *animalis* ATCC® 25527™ were obtained from KWIK-STIK™, Microbiologics, (MN, USA) and included in this study. The culture identities were confirmed using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) biotyper (Bruker, Bremen, Germany) by matching against the SARAMIS™ database for identification.

The lyophilised bacterial strains were subcultured in 10 ml sterile de Man, Rogosa and Sharpe media (MRS) (De Man et al., 1960) supplemented with L-cysteine (0.5 g/L) (MRS-C). The cultures were incubated anaerobically at 37 °C for 24 h in anaerobic jars made anoxic by anaerogen sachets (AnaeroGen™, Oxoid Ltd, Basingstoke, UK). The unadapted *Bifidobacterium* spp. were stored in 25% (v/v) sterile glycerol in cryotubes, as well as in cryobeads at -80 °C until needed.

When needed for use, the cultures were resuscitated in MRS-C broth under anaerobic conditions at 37 °C for 48 h unless specified otherwise. The culture broth or suspension media used for oxidative stress treatments was made anoxic by placing it inside an anaerobic jar with an anaerobic sachet directly after autoclaving.

4.2.2 Oxidative stress treatments

4.2.2.1 Hydrogen peroxide MIC

To select the H_2O_2 concentration for the adaptation treatments, the minimum inhibitory concentration (MIC) was first determined using the broth microdilution method described by Ibraheim et al. (2020), with modifications. Young colonies of each *Bifidobacterium* spp. grown on MRS-C agar at 37 °C for 24 h were used to prepare standardised bacterial suspensions of approximately 8 log CFU/ml (0.5 McFarland) in peptone buffered saline (PBS) (pH 7.3) with 0.05% L-cysteine (w/v). The standardised suspensions (2 μl) were used to inoculate 200 μl of MRS broth with varying H_2O_2 concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 mM H_2O_2) in 96-well microtitre plates. The plates were incubated anaerobically at 37 °C for 48 h. Bacterial growth was determined (OD_{600nm}) using the FLUOstar® Omega Microplate reader. Values were expressed as mean ± SE ($n = 3$).

The MIC was selected as the concentration of H_2O_2 at which no growth was visible. The MIC values were 0.4 mM H_2O_2 for *B. bifidum* and *B. breve* and 0.8 mM H_2O_2 for *B. animalis*. Therefore, for this study, the sublethal H_2O_2 concentration was set at 0.4 mM for *B. bifidum* and *B. breve* and 0.8 mM for *B. animalis*, and the lethal H_2O_2 concentration was set at 1 mM H_2O_2 for all three species.

4.2.2.2 H_2O_2 treatments

Cells of *B. bifidum*, *B. breve* and *B. animalis* were subjected to two H_2O_2 treatments. Firstly, mid-exponential cultures of each *Bifidobacterium* spp. (approximately 6 h at 37 °C, anaerobically) in anoxic de Man, Rogosa and Sharpe (De Man et al., 1960) (MRS) broth were exposed

to the sublethal H_2O_2 concentration (0.4 mM H_2O_2 for *B. bifidum* and *B. breve*; 0.8 mM H_2O_2 for *B. animalis*), as specified in the MIC, and incubated anaerobically for 48 h at 37 °C. Following incubation, surviving cells were spread-plated on MRS-C agar and incubated anaerobically for 24 h at 37 °C. Colonies were picked from the MRS-C agar plates as representative sublethal H_2O_2-treated cells and stored in cryotubes until further treatment and analysis.

Furthermore, the adaptation of *Bifidobacterium* spp. to oxidative stress was extended through the addition of a lethal H_2O_2 treatment. The sublethal H_2O_2-treated cells were grown on MRS-C agar and incubated anaerobically for 24 h. The cultures were then subjected to the lethal H_2O_2 treatment (1 mM H_2O_2) in anoxic MRS broth for 30 min at 37 °C. After the lethal H_2O_2 treatment, survivors were recovered by plating on MRS-C agar. Colonies recovered from this treatment were isolated as the first generation of lethal H_2O_2-treated variants. To manifest the response to oxidative stress permanently, the variants were subjected to two more generations of lethal H_2O_2 treatments. Colonies isolated from the third generation were preserved as the final representative lethal H_2O_2-treated *Bifidobacterium* variants for all subsequent experiments and were preserved in 25% glycerol (w/v) at -80 °C. Untreated cells, not exposed to H_2O_2, were used as controls in all analyses.

Prior to analysis, unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* spp. were resuscitated on MRS-C agar for 24 h incubating anaerobically at 37 °C. Colonies were picked and resuspended in anoxic double-distilled water (ddH_2O) to a cell count of approximately 8 log CFU/ml (0.5 McFarland).

4.2.3 Culture suspension preparation

Unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* spp. were anaerobically grown on MRS-C agar, incubating at 37 °C for 24 h. Resuscitated colonies were picked and resuspended in anoxic ddH_2O to a cell count of approximately 8 log CFU/ml (0.5 McFarland). Cell suspensions were prepared directly before analysis.

4.2.4 Flow cytometric analysis of membrane integrity or oxidation state

The effect of the adaptation treatments (sublethal- and lethal H_2O_2 treatments) on the membrane integrity and oxidation state was investigated using methods described by Fallico et al. (2020). The preprepared cell suspensions of *Bifidobacterium* spp. were stained with 6.68 μM SYTO 9 (S9) (Invitrogen™, Waltham, Massachusetts, USA) and 40 μM propidium iodide (PI) (Invitrogen™, Waltham, Massachusetts, USA) for the investigation of membrane integrity. Stained cell suspensions were incubated anaerobically at 37 °C for 15 min in the absence of light. Similarly, the sample preparation for the investigation of the intracellular oxidation state was performed by staining the cell suspensions with 0.5 μM CellROX® Green (CRG) (Invitrogen™, Waltham, Massachusetts, USA) before incubation under similar conditions.

Following the staining process, the samples were analysed by flow cytometry (FC) (BD Accuri Flow Cytometer, BD Biosciences, Belgium) at a flow rate of 35 μl/s, capturing 10,000 events per sample. The data collected was processed using FlowJo™ software version 10 (BD Life Sciences). Fluorescence of S9+ and CRG+ events was detected on the FL-1 channel (533/30 nm), and PI+ events were analysed on the FL-3 channel ($>$ 670 nm). Live and ethanol-treated dead (70% ethanol for 30 min), along with non-oxidised and oxidised (treated with 1 mM H_2O_2) *Bifidobacterium* spp. cells, were individually stained with S9, PI, CRG, and a combination of S9 and PI. These fluorescence signals were subsequently used to precisely identify and gate the relevant subpopulations of each FC assay. Additionally, for each sample analysed, the proportion of cells within each subpopulation was calculated and reported as a percentage of the overall cell count.

4.2.5 Electron microscopy

The unadapted and H_2O_2-treated *Bifidobacterium* cells were observed under a scanning and transmission electron microscope at the Central Laboratory for Microscopy and Microanalysis (University of Pretoria, Pretoria, South Africa), using the methods described by Booyens et al. (2014) with modifications. The preprepared cell suspensions were centrifuged at 1107 $\times$ g for 3 min using a Hermle Z 366 K centrifuge and washed twice with PBS (0.75 M, pH 7.4) for 15 min. The resulting cell pellets were fixed in 2.5% glutaraldehyde at 4 °C for 24 h.

Following fixation, the cells were rinsed thrice with PBS for 15 min before fixing with 1% osmium tetroxide (Merck, Germany).

After rinsing thrice with PBS for 15 min per rinse, the bacterial samples were fully dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and thrice in 100%) each for 15 min. For scanning electron microscopy (SEM), the dehydrated cell pellets were dried in a 50:50 mixture of hexamethyldisilazane (HMDS) (Merck, Germany) and 100% ethanol for 1 h. The dried cells were further suspended in HMDS for 1 h before spotting and drying on a glass coverslip overnight. The samples were mounted and coated with carbon before examining under a Zeiss Gemini Ultra Plus FEG SEM. The images were taken at 20 000 $\times$ magnification, and images with significant morphological characteristics as affected by the lethal challenge were selected for further analysis.

For transmission electron microscopy (TEM), the samples dehydrated in ethanol were resuspended in a 50:50 mixture of 100% ethanol and Quetol epoxy resin (Merck, Germany) for 1 h, which was followed by another suspension in epoxy resin for 1 h. The samples were then embedded in fresh resin and allowed to polymerise for 24 h at 60 °C. Ultrathin sections (0.5 μm) of the polymerised samples were stained with aqueous uranyl acetate (Merck, Germany) for 3 min as the primary stain and Reynold's lead citrate (Merck, Germany) as the counterstain for 3 min, rinsing with RO-H_2O between stains. The samples were viewed using a Jeol 2100F FEG TEM (JEOL, Tokyo, Japan), and images were selected based on significant ultrastructure characteristics affected by the H_2O_2 treatments. The Atlas of Bacterial & Archaeal Cell Structure (available at cellstructureatlas.org) was used to interpret the TEM images.

4.3 RESULTS

4.3.1 Gating for flow cytometry (FC)

Figure 4.1 shows the fluorescence histograms of live, membrane-intact (S9+) and ethanol-killed, membrane damage (S9-) control cells of *B. animalis*. Although the control cells were double stained with PI and S9, only the S9 fluorescence was used to determine the control gates. Both live and dead control cells showed high and indistinguishable PI signals. This

probably resulted from oxygen exposure before FC analysis, which was hard to exclude altogether. However, while the PI signal could not distinguish live and dead control cells, staining with S9 and PI allowed for a clear distinction between the subpopulations. Due to a greater nucleic acid binding affinity of PI, cells with compromised cell membranes showed a lower S9 fluorescence upon double staining. This gate is shown as 'Damaged Membrane (S9-)' in Figure 4.1. Similarly, cells with intact membranes showed a high S9 fluorescence upon double staining. This gate is shown as 'Intact Membrane (S9+)' (Figure 4.1).

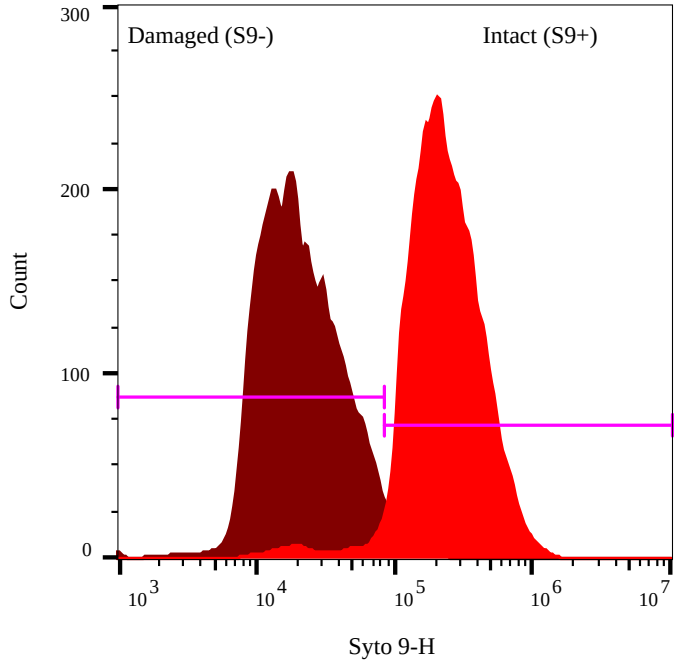


Figure 4.1 SYTO 9 fluorescence histogram of *B. animalis* control cells.

Figure 4.2 shows the fluorescence histograms of control cells of *B. animalis* stained with CRG. Live and unstained cells were used as the control to identify unoxidised cells (N-ROS) and were set just above 10^2 AU. Due to the inevitable exposure to oxygen during analysis, live cells stained with CRG were used to gate for cells in a low-oxidation state (L-ROS+). Live cells exposed to the lethal dose for oxidative stress were used to gate for cells with a high oxidation state (H-ROS+).

The fluorescence signal profiles observed in control samples of *B. animalis*, considering their unavoidable exposure to oxygen during analysis, served as the basis for establishing the measurement gates across all three *Bifidobacterium* strains. The gating strategy was consistently applied to FC analyses of *B. bifidum*, *B. breve*, and *B. animalis* throughout the study.

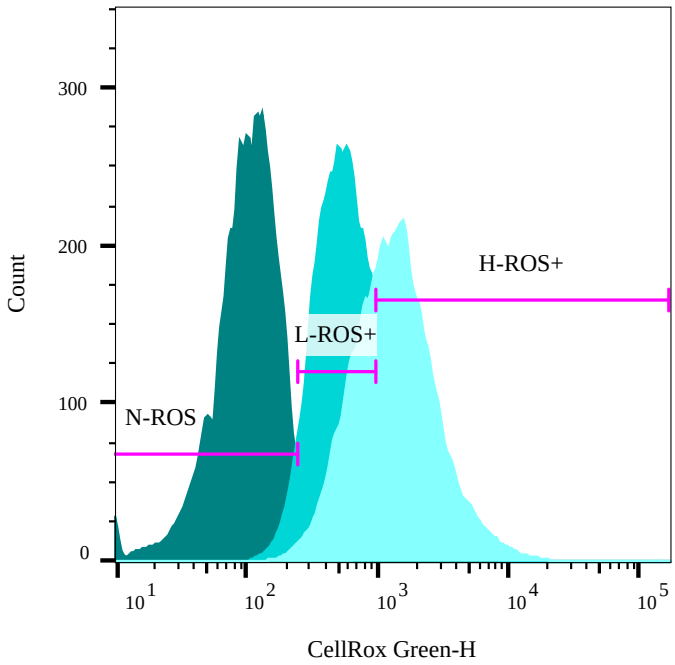


Figure 4.2 CellRox Green fluorescence histogram of *B. animalis* control cells. N-ROS represents the unoxidised cell gate, L-ROS+ represents a low-oxidised cell state, and H-ROS represents a highly-oxidised cell state.

4.3.2 Assessment of membrane integrity of H_2O_2-treated *Bifidobacterium* spp.

The three *Bifidobacterium* spp. presented with intact membranes, except for the unadapted *B. bifidum* cells (Figure 4.3 (A)). The majority of the unadapted *B. bifidum* population was observed fluorescing within the gate indicative of membrane damage (S9-) (77%) (Figures 4.3 (A) and 4.4). In contrast, only a minor fraction of the H_2O_2-treated *B. bifidum* cells showed signs of membrane damage (S9-) (19%), with no observable difference between sublethal- and lethal H_2O_2-treated cells (Figures 4.3 (A) and 4.4).

Similarly, H_2O_2-treated *B. breve* cells displayed intact cell membranes (S9+), as shown in Figure 4.3 (B). However, the sublethal H_2O_2-treated *B. breve* cells showed greater membrane integrity compared to lethal H_2O_2-treated (91% vs. 87%, respectively) (Figure 4.4). In contrast, unadapted *B. breve* cells exhibited lower S9 fluorescence, indicating damaged membranes, where 42% of the unadapted *B. breve* cells presented with damaged membranes (S9-) (Figures 4.3 (B) and 4.4).

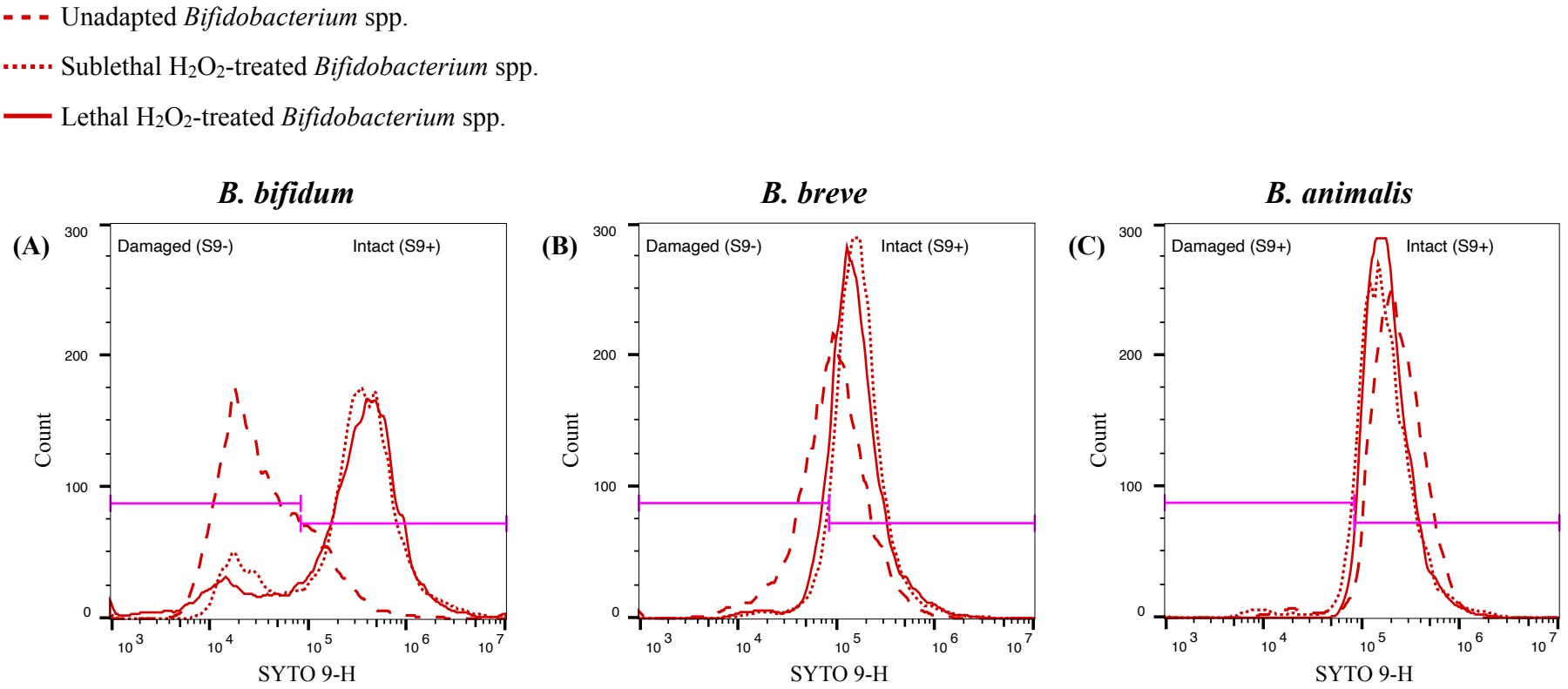


Figure 4.3 SYTO 9 fluorescence histograms of unadapted, sublethal- and lethal H_2O_2-treated (A) *B. bifidum*, (B) *B. breve* and (C) *B. animalis*. S9-: damaged membrane cell gate; S9+: intact membrane cell gate.

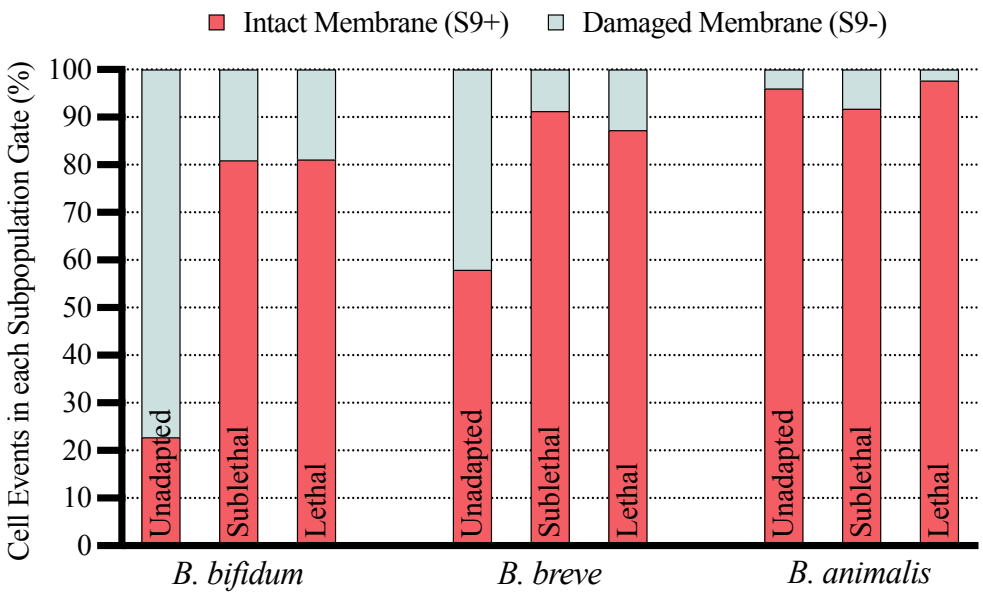


Figure 4.4 Relative proportions of membrane intact (S9+) and membrane damaged (S9-) subpopulations of unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* species.

Interestingly, the S9 fluorescence histogram for *B. animalis* cells did not show a clear fluorescence pattern differentiating the unadapted, sublethal-, and lethal H_2O_2-treated cells (Figure 4.3 (C)). However, Figure 4.4 clarified that lethal H_2O_2-treated *B. animalis* cells had superior membrane integrity compared to both the unadapted and sublethal H_2O_2-treated cells, as well as among the three *Bifidobacterium* species examined in this study.

4.3.3 Intracellular oxidation state of H_2O_2-treated *Bifidobacterium* spp.

The fluorescence data in Figure 4.5 indicated that all three *Bifidobacterium* species were in an oxidised state, regardless of H_2O_2 treatment. Notably, the H_2O_2-treated *Bifidobacterium* spp. tended to exhibit increased CRG fluorescence, indicating cells with a highly oxidised intracellular state (H-ROS+). This was particularly observed in the lethal H_2O_2-treated cells (Figure 4.5).

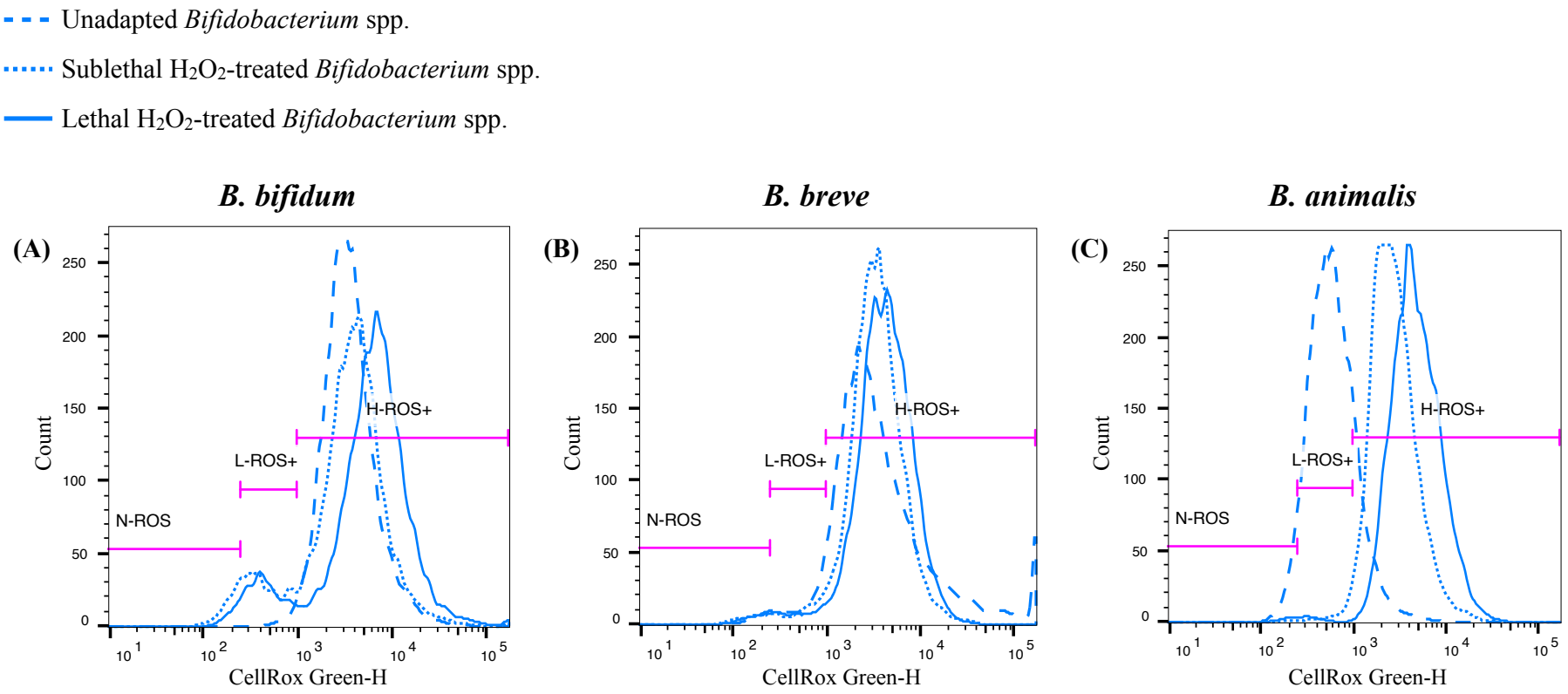


Figure 4.5 CellRox Green fluorescence histograms of unadapted, sublethal-, and lethal H_2O_2-treated (A) *B. bifidum*, (B) *B. breve* and (C) *B. animalis*. N-ROS: Unoxidised cell gate; L-ROS+: Low-oxidised cell gate; H-ROS+: High-oxidised cell gate.

Interestingly, unadapted *B. bifidum* showed a lower CRG fluorescence peak in Figure 4.5 (A) compared to H_2O_2-treated cells. However, upon closer inspection, Figure 4.6 highlighted the majority of the unadapted *B. bifidum* population as highly oxidised (H-ROS+) (99%), surpassing the levels observed in both sublethal- and lethal H_2O_2-treated cells (85% and 89%, respectively). This discrepancy was attributed to a small proportion of the H_2O_2-treated *B. bifidum* cells displaying low CRG fluorescence (Figure 4.5 (A)). Subsequently, 11% and 10% of the sublethal- and lethal H_2O_2-treated *B. bifidum* cells, respectively, were in a lowly oxidised state (L-ROS+), with 4% and 2%, presenting as unoxidised (L-ROS+) (Figure 4.6).

Unadapted *B. breve* cells demonstrated lower CRG fluorescence compared to H_2O_2-treated counterparts (Figure 4.5 (B)). However, there were no notable differences in subpopulation densities among unadapted, sublethal-, and lethal H_2O_2-treated *B. breve* cells.

Among the H_2O_2-treated *Bifidobacterium* spp., unadapted *B. animalis* exhibited the majority of their population within the low-oxidised subpopulation gate (L-ROS+) (76%), as evidenced by their low CRG fluorescence in Figure 4.5 (C). This result is also evident in Figure 4.6. Conversely, both sublethal- and lethal H_2O_2-treated cells exhibited higher intracellular oxidation states, whereby lethal H_2O_2-treated cells showed the highest proportion in a highly oxidised state (H-ROS+) (98%) (Figures 4.5 (C) and 4.6).

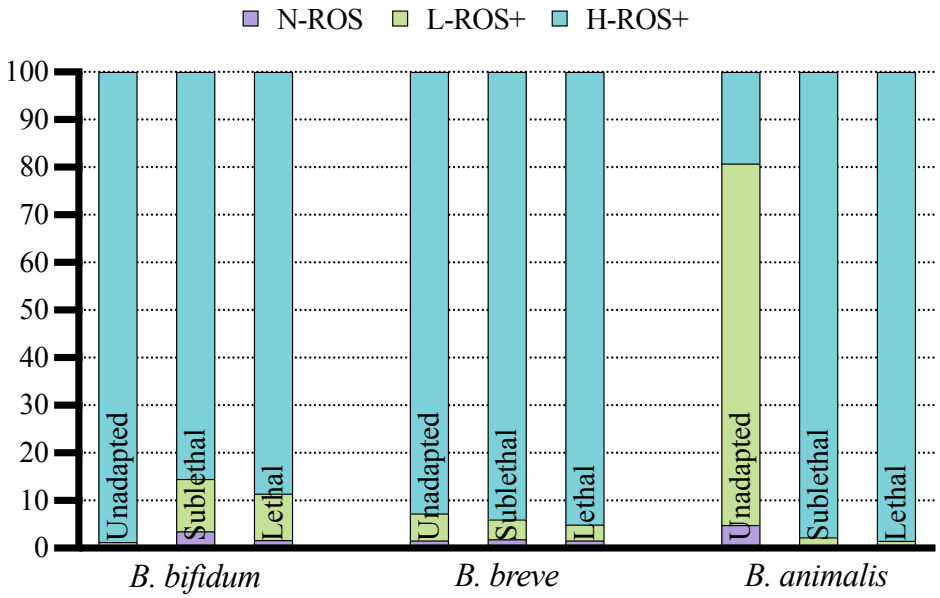


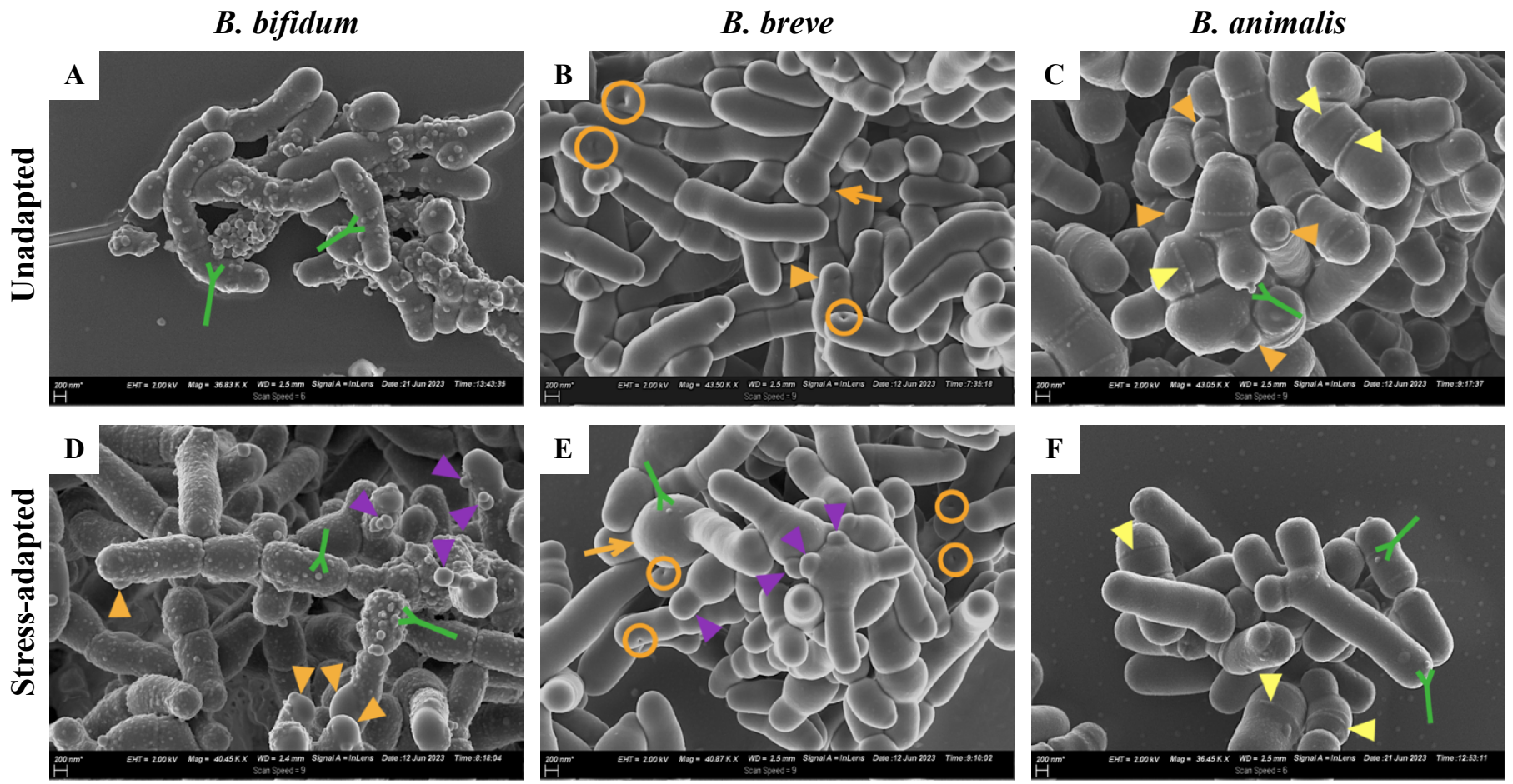
Figure 4.6 Relative proportions of unoxidised (N-ROS), low-oxidised (L-ROS+) and highly-oxidised (H-ROS+) subpopulations of unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* species.

4.3.4 Morphology of H_2O_2-treated *Bifidobacterium* spp.

Figure 4.7 depicts the morphological characteristics and responses of unadapted and H_2O_2-treated *Bifidobacterium* species. Notably, the morphological adaptations to the H_2O_2 treatment were observed to be specific for each species. Regardless of the H_2O_2 treatment, *B. bifidum* cells consistently showed the presence of cell surface structures typical of extracellular vesicles (EVs) (Figures 4.7 (A) and (D)). These structures gave the cells a distinctly rough outer surface. In contrast, *B. breve* (Figures 4.7 (B) and (E)) and *B. animalis* (Figures 4.7 (C) and (F)) had relatively smoother cell surfaces. However, *B. animalis* cells, particularly the H_2O_2-treated cells, displayed prominent circumferential rings, as indicated in Figures 4.7 (C), and (F). H_2O_2-treated *B. bifidum* cells (Figure 4.7 (D)) appeared as shorter rods than their unadapted counterparts (Figure 4.7 (A)). Similarly, H_2O_2-treated *B. breve* and *B. animalis* displayed shortened rods (Figures 4.7 (E) and (F), respectively). Notably, H_2O_2-treated *B. breve* cells displayed more pronounced coccoid ends (Figure 4.7 (E)). In H_2O_2-treated *B. bifidum* (Figure 4.7 (D)) and *B. animalis* (Figure 4.7 (C) and (F)), smooth protrusions and budding of coccoid cells were observed.

4.3.5 Ultrastructure of H_2O_2-treated *Bifidobacterium* spp.

Figure 4.8 depicts the intracellular ultrastructure characteristics and responses of unadapted and H_2O_2-treated *Bifidobacterium* species. Overall, each *Bifidobacterium* species exhibited a distinct reorganisation of its internal structures due to the H_2O_2 treatment. Before treatment, the cytoplasm of unadapted cells of *B. breve* and *B. animalis* appeared more homogenous (Figures 4.8 (B) and (C), respectively), contrary to *B. bifidum* (Figure 4.8 (A)). However, H_2O_2-treated *B. animalis* displayed a consistent and smooth cytoplasmic distribution (Figure 4.8 (F)). H_2O_2-treated *B. bifidum* cells had fewer ribosomes than unadapted cells (Figures 4.8 (D) and (F), respectively), while H_2O_2-treated *B. animalis* showed an increase in ribosomes (Figure 4.8 (F)).



(≺ — outer membrane vesicles; ○ — pores; → — cellular swelling; ▶ — cellular protrusions; ▶ — circumferential rings; ▫ — irregular cell envelope/ cell damage; ▶ — coccoid progeny).

Figure 4.7 Scanning electron images of unadapted and H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* cells.

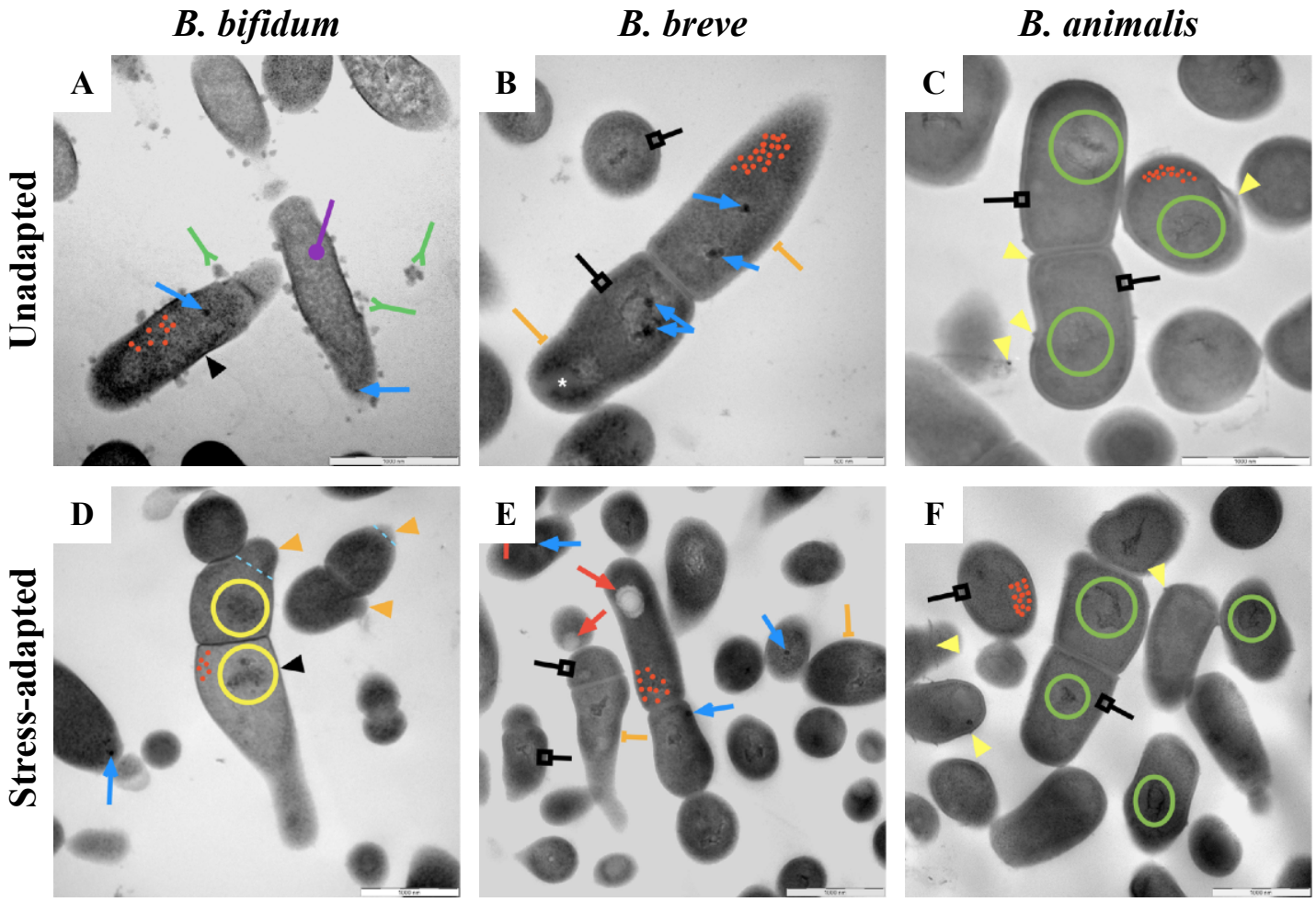


Figure 4.8 Transmission electron images of unadapted and H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* cells.

4.4 DISCUSSION

Previous research demonstrated that exposure to sublethal doses of H_2O_2 temporarily increased bacterial tolerance to oxidative stress (Oberg et al., 2011) However, these improvements were observed to be transient. Therefore, in this study, the stress adaptation treatment was extended through the subsequent and repeated exposure to lethal concentrations of H_2O_2, with the aim of permanently manifesting the induced oxidative stress response in the bacteria. This study aimed to characterise the physiological states of three *Bifidobacterium* species following sublethal- and lethal H_2O_2 treatments.

4.4.1 Improved membrane integrity of H_2O_2-treated *Bifidobacterium* spp.

The findings of membrane integrity highlight the intrinsic sensitivity of *B. bifidum* to environmental stress factors compared to the other two species included in this study (Schöpping et al., 2022). The high proportion of damaged membranes observed in *B. bifidum* was likely due to unavoidable exposure to oxygen and mechanical damage during FC analysis (Kawasaki et al., 2018*a*; Rosenberg et al., 2019).

The intact cell membranes of the majority of *B. breve* and *B. animalis* reflect their greater intrinsic tolerance to oxidative stress, compared to *B. bifidum*. According to the revised classification suggested by Kawasaki et al. (2018*a*), the *Bifidobacterium* species included in this study are classified as O_2-sensitive, whereby this bacteria is able to tolerate O_2 concentrations of 5%. However, the suggested classification for aerotolerance of *Bifidobacterium* does not eradicate differences in tolerance to oxidative stress between species within this group, as is evident from the results.

A higher proportion of cells with intact membranes in the H_2O_2-treated *Bifidobacterium* populations, compared to the unadapted cells, was likely due to modifications in the membrane composition resulting from the H_2O_2 treatments. A study by Oberg et al. (2013) highlighted an augmented incorporation of fatty acids into the lipid membranes of *Bifidobacterium* spp. in the presence of H_2O_2 stress, a change correlated with an enhanced resilience to H_2O_2 exposure in a particular *Bifidobacterium* strain. The incorporation of fatty acids into the bacterial cell mem-

brane has been shown to affect its fluidity and permeability (Mohedano et al., 2022). Therefore, it is possible that the increase in membrane integrity of H_2O_2-treated *Bifidobacterium* cells in this study was due to an enhancement of fatty acid incorporation into the cell membrane and, subsequently, reduced membrane fluidity and permeability. Furthermore, these findings are supported by the high S9 fluorescence observed in H_2O_2-treated *Bifidobacterium* cells, indicating that a bacterial cell membrane with diminished permeability effectively prevents PI from penetrating the cells and substituting the membrane-permeable S9 dye.

Intriguingly, the enhanced membrane integrity of *B. breve* cells after sublethal- versus lethal H_2O_2 treatments likely reflects the threshold of stress these cells can tolerate before cellular mechanisms are overwhelmed. In contrast, *B. animalis* might exhibit a contrasting pattern of resilience due to different inherent adaptive responses and membrane robustness. The differential behaviour between these two species under the sublethal- and lethal H_2O_2 treatments could be attributed to distinct genetic and physiological pathways that govern their stress response mechanisms (Schöpping et al., 2022). For instance, *B. breve* might not efficiently upregulate protective mechanisms under extreme oxidative conditions, leading to greater membrane damage. This hypothesis suggests that the extent of damage and the ability to maintain membrane integrity might be closely tied to the specific adaptive capacities of each species. Nevertheless, additional research is essential to explore and confirm the changes in membrane composition associated with the stress adaptation treatments.

4.4.2 Oxidation states increased in H_2O_2-treated *Bifidobacterium* spp.

All three *Bifidobacterium* spp. presented with high intracellular oxidation states, a condition that appears to be consistent across different H_2O_2 treatments. This consistent pattern suggests that the oxidation state may be an intrinsic characteristic of these species rather than solely a response to external H_2O_2 exposure. Notably, *B. bifidum*, even in its unadapted state, was in a highly oxidized state, likely exacerbated by the inevitable oxygen exposure during the FC analysis. This observation underlines the O_2-sensitive nature of *B. bifidum* and underscores its particular vulnerability to oxidative stress (Kawasaki et al., 2018*a*). These characteristics emphasise the need for careful handling and specific protective measures when conducting research with *Bifidobacterium* spp., particularly *B. bifidum*.

Interestingly, a subset of H_2O_2-treated *B. bifidum* cells exhibited a significantly lower intracellular oxidation state. Recent work has identified and characterised a TrxR enzyme that exerts H_2O_2 detoxification activities (Satoh et al., 2019). This suggests the possibility that the H_2O_2 treatment resulted in the sustained activation of this enzyme in a minor subpopulation of *B. bifidum*, leading to their notably reduced oxidation levels as detected by FC. Future research should aim to verify whether this subpopulation indeed exhibits increased tolerance to oxidative stress.

During the investigation of the oxidation state of *B. breve*, no distinct differences in the intracellular oxidation states were observed between the unadapted and H_2O_2-treated cells, which may indicate effective detoxification mechanisms of *B. breve*, suggesting that the cells can effectively neutralise H_2O_2 and restore its redox balance during adaptation treatments. To validate these preliminary observations, further research focusing on the stress response of H_2O_2-treated *B. breve* cells is warranted. Moreover, *B. breve* may employ alternative stress response mechanisms that were undetectable during the FC analysis.

Overall, the unadapted *B. animalis* cells exhibited the lowest intracellular oxidation state, indicative of their intrinsic tolerance to oxidative stress. Likewise, Oberg et al. (2013) noted that *B. animalis* subsp. *lactis* BL-05 exhibited greater inducible H_2O_2 resistance compared to *B. longum* NCC2705. Such comparison underscores the variability of intrinsic and adaptive H_2O_2 defences across different species and strains within the *Bifidobacterium* genus.

4.4.3 Morphological adaptations of H_2O_2-treated *Bifidobacterium* spp.

Specific morphological changes may be associated with the adaptive stress response and may be evident in the FC analysis (Ruiz et al., 2007). The findings from the SEM analysis revealed the production and release of EVs to be consistently expressed by *B. bifidum* irrespective of adaptation, which resulted in a textured surface. In line with these findings, Bose et al. (2020) classified EVs released by Gram-positive bacteria to contain fatty acids, phospholipids, peptidoglycan, cytoplasmic proteins, and membrane-associated molecular chaperones. The rough cell surface observed might reflect not just EV release, but also disrupted cell division and changes in cell wall composition or structure, a possible negative effect of oxidative stress

(Ahn et al., 2001; Mozaheb and Mingeot-Leclercq, 2020). Furthermore, Qian et al. (2011) observed the formation of intracellular granules in *Bifidobacterium* spp. grown in MRS without a reducing agent, suggesting that the development of vesicle-like structures is integral to the stress response mechanism of *Bifidobacterium* species. The exact composition of EVs of *Bifidobacterium* spp. has not been studied intensely and requires further investigation. However, one study found that *B. longum* NCC2705 releases EVs rich in cytoplasmic proteins, including mucin-binding proteins, which may promote bacterial adhesion and survival in the gastrointestinal tract (Nishiyama et al., 2020).

Interestingly, the protruding circumferential rings observed in *B. animalis* in this study were also observed in a surface analysis conducted by Shang et al. (2013) using atomic force microscopy (AFM) of *B. animalis* RH (Shang et al., 2013). The researchers describe the circumferential rings as 'large worm-like or ring-like structures' and were hypothesised to be tangled networks of EPS potentially involved in cell aggregation (Shang et al., 2013).

4.4.4 H_2O_2-treated *Bifidobacterium* spp. display distinct ultrastructural adaptations

The findings from the TEM analysis revealed that the adaptation treatment resulted in minimal intracellular differences in *B. animalis* cells, although it was observed to affect the cytoplasmic homogeneity in *B. breve*. During the stress adaptation treatment, the cellular components in the *B. breve* cells, including DNA, organelles, ribosomes, and proteinaceous structures, likely clustered together, reducing their surface area exposed to oxidative stress (Rahman et al., 2008). This compaction strategy could serve as a defence mechanism, limiting the exposure of these structures to potential damage from oxidative stress.

Furthermore, the general granular cytoplasm of *B. bifidum* could be due to the presence of poly P granules (Qian et al., 2011), which are orthophosphate residues that form polyanionic inorganic biopolymers in response to oxidative stress (Kornberg, 1995). Similar observations were made for the oxidative stress response of *B. scardovii* (Qian et al., 2011). These results suggest a potential role of these internal granules in the response of *B. bifidum* to oxidative stress, though further research is needed for clarification and confirmation.

4.5 CONCLUSION

The main findings of this study highlight the species-specific adaptive mechanisms of *Bifidobacterium* spp. to oxidative stress, revealing significant insights into their survival strategies. The H_2O_2-treated *Bifidobacterium* spp. show distinct patterns of membrane integrity, with notable improvements in resilience against oxidative damage following H_2O_2 treatment. This is especially true for *B. bifidum*, suggesting an adaptive enhancement in membrane structure possibly through changes in fatty acid composition. Moreover, differential intracellular oxidation states underscore the inherent tolerance to oxidative stress of *B. animalis*. Sublethal and lethal H_2O_2 treatments result in morphological and ultrastructural adaptations, including the strategic production and release of EVs and the emergence of poly P granules in *B. bifidum*. Additionally, these treatments lead to the compaction of cellular components in *B. breve*, all of which are crucial adaptive responses to oxidative challenges.

Future research should delve into the effects of the H_2O_2 treatments on *Bifidobacterium* spp. tolerance to oxidative stress. This exploration will deepen the understanding of their stress response and facilitate the selection of stress-adapted strains for enhanced probiotic applications, particularly during yoghurt production.

CHAPTER 5

Investigating the stress response of stress-adapted *Bifidobacterium* spp.

This study investigated the physiological and morphological characteristics, growth responses, and kinetics of the stress-adapted *Bifidobacterium* spp. variants when subjected to a lethal H_2O_2 challenge, with the aim of isolating variants of *Bifidobacterium* spp. with enhanced tolerance to lethal levels of oxidative stress. Unadapted, sublethal and lethal H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis* cells were subjected to a lethal H_2O_2 challenge before analysis. Flow cytometry (FC) analysis using CellROX® Green (CRG) and propidium iodide (PI) indicated that the lethal H_2O_2 treatment improved membrane integrity and oxidative stress resilience, with species-specific variations. *B. animalis* showed significant membrane integrity and reduced oxidation states under lethal challenge, demonstrating an innate ability to adapt its membrane composition effectively. Growth kinetic analyses showed *B. breve* consistently thriving across varying H_2O_2 levels, suggesting unique resilience mechanisms, whereas *B. bifidum* and *B. animalis* displayed increased growth with higher H_2O_2 concentrations, likely due to enhanced hydrophobicity and autoaggregation. SEM and TEM revealed extracellular vesicle formation and morphological changes like cell elongation and surface texturing, suggesting defensive adaptations against oxidative stress. The results suggest that oxidative stress adaptation in *Bifidobacterium* spp. can improve its resilience to oxidative stress during yoghurt manufacture, with better adaptation in *B. animalis* possibly related to its genetic predisposition.

Keywords: *Bifidobacterium* spp., oxidative stress response, stress adaptation, flow cytometry, membrane integrity and oxidation state, growth kinetics, morphology

5.1 INTRODUCTION

Bifidobacterium spp. are natural inhabitants of the human GIT of healthy humans, making up 10 - 40% of the full microbiota composition (Arboleya et al., 2016). The incorporation of *Bifidobacterium* spp. into one's diet is linked to mitigating the effects of gut dysbiosis, facilitating the upkeep of a more balanced gut microbiome and its functions, and providing therapeutic advantages to the host (He et al., 2023). Given these beneficial outcomes, *Bifidobacterium* spp. has garnered popularity as a potential probiotic for incorporation into the formulation of new, functional food products (He et al., 2023). As per the definition of probiotics, *Bifidobacterium* spp. need to be 'live' and 'administered in sufficient quantities' to be able to 'confer a health benefit to the host' (FAO/WHO, 2002; Hill et al., 2014). For probiotics to yield therapeutic benefits, it is recommended that the food product contains a minimum of 6 log CFU/g of viable cells at the time of consumption (FAO/WHO, 2003; Nyanzi et al., 2021). Yet, incorporating *Bifidobacterium* spp. in foods is challenging, owing to its decline in viability to below the recommended therapeutic dose during shelf-life (Sibanda et al., 2024).

Among the many potential probiotic carrier foods, dairy foods, particularly yoghurt, are optimal vehicles for delivering probiotics owing to their widespread popularity and distinctive matrix with a buffering capacity (Hadjimbei et al., 2022). However, several stress factors associated with the processing of yoghurt can negatively affect *Bifidobacterium* spp. viability. These stress factors that exert adverse effects on *Bifidobacterium* spp. viability include acid, osmotic, heat, cold, and oxidative stress (Sibanda et al., 2024). As anaerobes, *Bifidobacterium* spp. are intrinsically susceptible to exogenously and endogenously generated reactive oxygen species (ROS) such as H_2O_2. Due to the absence of genes encoding for enzymes typically associated with aerobic respiration in microorganisms (i.e. SOD, catalase or NADH peroxidase), most species of bifidobacteria are unable to fully reduce oxygen into harmless compounds (Zuo et al., 2014).

The exposure of probiotic microorganisms to stressful conditions has been observed to elicit an adaptive response (Schöpping et al., 2022). The primary response mechanisms to oxidative stress of *Bifidobacterium* spp. are based on the production of enzymes that can detoxify H_2O_2 and ROS, such as AhpC and TrxR (Oberg et al., 2015; Schöpping et al., 2022), together with protective activities of several chaperones and reparative proteins (Zuo et al., 2018). Some

of the enzymes associated with the adaptive response to oxidative stress can be induced and manifested in the bacterium permanently through a process of stress adaptation (Mills et al., 2011). The process of stress adaptation of *Bifidobacterium* spp. involves the induction of the stress response without a loss in viability through pre-exposure to a sublethal dose of the stress (Fiocco et al., 2020). To permanently establish this stress response in the bacterium, the process involves multiple subsequent cultivations under lethal stress conditions, with survivors being isolated and subjected to further exposure after each cycle (Jiang et al., 2016). Using a similar approach, an improved tolerance to lethal H_2O_2 concentrations was observed in various strains of *Bifidobacterium* spp. (Oberg et al., 2011).

In the preceding chapter of this thesis (Chapter 4), the physiological, morphological and ultrastructural characteristics of *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis* subjected to a stress adaptation treatment were assessed. Building on the findings of the previous chapter, the objective of this study was to investigate the physiological, morphological and ultrastructural characteristics, as well as growth responses and kinetics, of the stress-adapted *Bifidobacterium* spp. variants when subjected to a lethal H_2O_2 challenge, with the aim of isolating variants of *Bifidobacterium* spp. with enhanced tolerance to lethal levels of oxidative stress.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial cultures used in the study

The bacterial cultures used in this study were *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis*, which were subjected to stress adaptation treatments as described in Chapter 4(Section 4.2.2). The storage and cultivation conditions for these cultures were consistent with those outlined in Chapter 4 (Section 4.2.1).

5.2.2 Culture suspension preparation and lethal H_2O_2 challenge

Cultures of unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* cells (Chapter 4) were anaerobically grown on MRS-C agar for 24 h at 37 °C before resuspending in sterile

and anoxic ddH_2O to a cell count of 8 log CFU/ml (0.5 McFarland). Double-distilled water (ddH_2O) was made anoxic by placing it in anaerobic jars with anaerobic sachet directly after autoclaving for at least 24 h before treatments. The bacterial cell suspensions were subjected to a lethal challenge directly before analysis. The lethal challenge involved the addition of H_2O_2 to each cell suspension up to a concentration of 1 mM, followed by anaerobic incubation at 37 °C for 30 min.

5.2.3 Flow cytometric analysis of membrane integrity and oxidation state

Cell suspensions of unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* cells were immediately stained and analysed by FC following the lethal challenge. The samples were stained first with 0.5 μM CellROX® Green (CRG) (Invitrogen™, Waltham, Massachusetts, USA) for 15 min at 37 °C under conditions of anaerobiosis and darkness to prevent exposure to oxygen and light. The samples were then stained with 8 μM propidium iodide (PI) (Invitrogen™, Waltham, Massachusetts, USA) for 15 min under similar conditions as for CRG staining.

After staining, samples were analysed by FC (BD Accuri Flow Cytometer, BD Biosciences, Belgium) at a flow rate of 35 μl/s, and 10000 events were collected for each sample. Data acquired from the FC analysis was processed by FlowJo™ v10.8.1 Software (BD Life Sciences). Fluorescence of CRG+ events was detected on the FL-1 channel (533/30 nm), and PI+ events were analysed on the FL-3 channel ($>$ 670 nm). Live and dead (treated with 70% ethanol; 30 min), as well as unoxidised and oxidised (treated with 1 mM H_2O_2) cells of *Bifidobacterium* spp., were stained separately with PI, CRG, and CRG plus PI. The respective fluorescence signals of each sample were then utilised to accurately gate for the relevant bacterial subpopulations. Furthermore, cell event counts within these subpopulations for each bacterial sample were extracted and presented as a percentage of the total cell population.

5.2.4 Choice of stress-adapted *Bifidobacterium* variants

As the stress adaptation treatment involved two H_2O_2 treatments (Chapter 4), a stress-adapted variant had to be chosen for subsequent analyses based on the results from the FC analysis. Consequently, the lethal H_2O_2-treated variants of all three *Bifidobacterium* species were chosen for subsequent analysis in this study and will henceforth be referred to as the *stress-adapted variants*.

5.2.5 Growth kinetics

The growth kinetics of the unadapted and stress-adapted variants of *Bifidobacterium* spp. were determined by a turbidimetric method (OD_{600nm}) using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany). An 8 log CFU/ml (0.5 McFarland) suspension of each unadapted and stress-adapted *Bifidobacterium* spp. was prepared. Each bacterial suspension was inoculated (2 μl) into wells containing anoxic MRS broth (200 μl) with either 0.0, 0.1 or 1.0 mM initial H_2O_2 concentrations. The optical density of each well was measured at six hour intervals over a 36 h anaerobic incubation period (0, 6, 12, 24, and 36 h) at 37 °C. The growth rate under the varying levels of initial H_2O_2 concentrations was calculated using the following equation:

$$\mu\,(\mathrm{h}^{-1}) = \frac{\ln(\mathrm{OD}_{12h}/\mathrm{OD}_{0h})}{12},$$

where μ is the growth rate (h^{-1}), OD_{12h} is the optical density at 12 h, and OD_{0h} is the optical density at 0 h. An exponential growth phase was observed for all samples within the first 12 hours and was hence used in the growth rate calculations. The maximum population density (MPD) was determined as the optical density (OD) of each control and stress-adapted *Bifidobacterium* spp. after 36 h of incubation. Duplicate samples were prepared and the growth kinetics analysis was repeated once ($n = 4$).

5.2.6 Electron microscopy

The unadapted and stress-adapted *Bifidobacterium* variants were observed under a SEM and TEM after exposure to the lethal challenge. The preparation and analysis of the *Bifidobacterium* cells were conducted using the method described in Chapter 4 (Section 4.2.5).

5.2.7 Statistical analysis

Differences in growth rates and MPDs among the unadapted and stress-adapted *Bifidobacterium* variants under varying H_2O_2 concentrations were analysed using a two-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) Version 29 software (IBM, USA). A Tukey's multiple comparisons test was performed with the same statistical software to determine any statistically significant differences in growth kinetics among the *Bifidobacterium* species. A significance level of 0.05 was used in all analyses.

5.3 RESULTS

5.3.1 Gating for flow cytometry

The fluorescence gates were set using control cells of *B. animalis* that were double stained with CRG and PI. The biplot of this gating, shown in Figure 5.1, resulted in four gates, defined as follows: N-ROS; PI- cells were unoxidised and maintained their membrane integrity and viability; N-ROS; PI+ cells were classified as dead cells, with a complete loss of cell integrity; ROS+; PI+ cells were classified as cells that in an oxidised state, exhibiting characteristics associated with membrane damage; ROS+; PI- cells were classified as cells that maintained their membrane integrity while in an oxidised intracellular state.

The fluorescence signal profiles observed in control samples of *B. animalis*, particularly considering their unavoidable exposure to oxygen during analysis, served as the basis for establishing the measurement gates across all three *Bifidobacterium* species. The gating strategy was consistently applied to FC analyses of *B. bifidum*, *B. breve*, and *B. animalis* throughout the study.

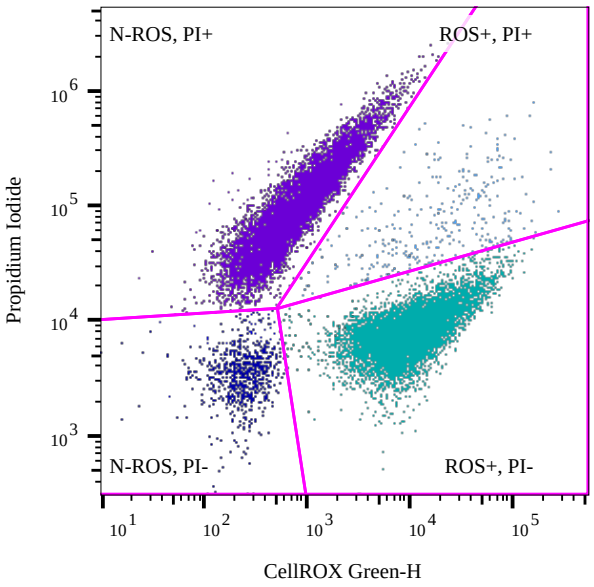


Figure 5.1 CellRox Green and propidium iodide fluorescence biplot of *B. animalis* control cells. N-ROS, PI- gate represents unoxidised cells with an intact membrane, N-ROS, PI+ gate represents unoxidised cells with a damaged membrane, ROS+, PI- gate represents oxidised cells with an intact membrane, ROS+, PI+ gate represents oxidised cells with a damaged membrane.

5.3.2 Combined assessment of membrane integrity and intracellular oxidation state in stress-adapted *Bifidobacterium* spp. under lethal H_2O_2 challenge.

As observed in Figure 5.2, each *Bifidobacterium* species displayed a unique response to the lethal H_2O_2 challenge in terms of maintenance of membrane integrity and control of intracellular oxidation state. The majority of each *Bifidobacterium* population presented with an intact membrane in an oxidised state (ROS+, PI-) following the lethal challenge, except for the unadapted *B. bifidum* cells (Figures 5.2 (A) and 5.3). Most of the unadapted *B. bifidum* cells exhibited membrane damage whilst in an unoxidised state (N-ROS, PI+) (38.6%) (Figures 5.2 (A) and 5.3), which alludes to the subpopulation gate that is classified as dead cells

resulting from ROS accumulation, as shown in Section 5.3.1. In contrast, H_2O_2-treated cells of *B. bifidum*, both sublethal- and lethal H_2O_2-treated, exhibited the greatest proportion of their population with an intact membrane whilst in an oxidised state (ROS+, PI-) (45.3% and 44.4%, respectively) (Figures 5.2 (D), (G) and 5.3).

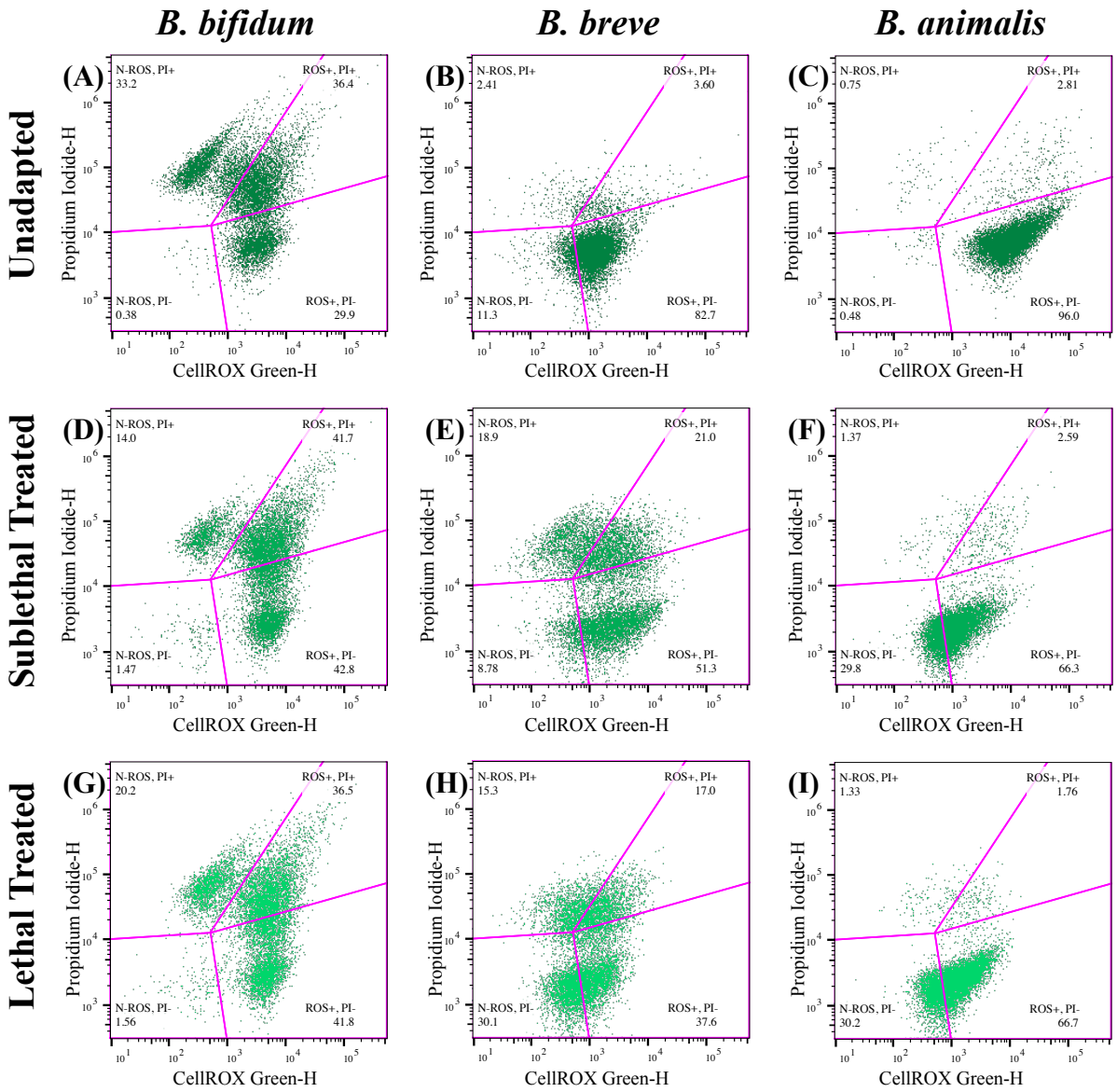


Figure 5.2 CellRox Green and propidium iodide fluorescence biplots of unadapted, sublethal- and lethal H_2O_2-treated *B. bifidum*, *B. breve* and *B. animalis* following exposure to the lethal H_2O_2 challenge (1 mM H_2O_2; 30 min). N-ROS, PI+: Unoxidised, membrane-damaged cell gate; N-ROS, PI-: Unoxidised, membrane-intact cell gate; ROS+, PI+: Oxidised, membrane-damaged cell gate; ROS+, PI-: Oxidised, membrane-intact cell gate.

It is evident in Figure 5.2 (B) that the unadapted *B. breve* cells demonstrated superior retention of membrane integrity than their H_2O_2-treated counterparts following exposure to the lethal H_2O_2 challenge. This observation is also evident in Figure 5.3, whereby 83.8% of the unadapted *B. breve* cells fluoresced within the (ROS+, PI-) subpopulation gate. However, the lethal H_2O_2-treated *B. breve* cells demonstrated greater control of intracellular oxidation state compared to the sublethal H_2O_2-treated cells, as illustrated by the bi-fluorescence of CRG and PI within the (N-ROS, PI-) subpopulation gate in Figure 5.2 (H).

Among the three *Bifidobacterium* spp., *B. animalis* retained the highest proportion of oxidised cells with intact membranes (ROS+, PI-), irrespective of adaptation or oxidation state (Figures 5.2 (C), (F) and (I)). However, a greater extent of sublethal- and lethal H_2O_2-treated *B. animalis* cells (28.9% and 29.7%, respectively) presented with an unoxidised intracellular state and intact membrane (N-ROS, PI-) (Figures 5.2 (F) and (I)), than the unadapted cells (0.%) after the lethal challenge (Figures 5.2 (C) and 5.3). This result was distinctly more evident for the lethal H_2O_2-treated *B. animalis* cells (Figure 5.2 (I)).

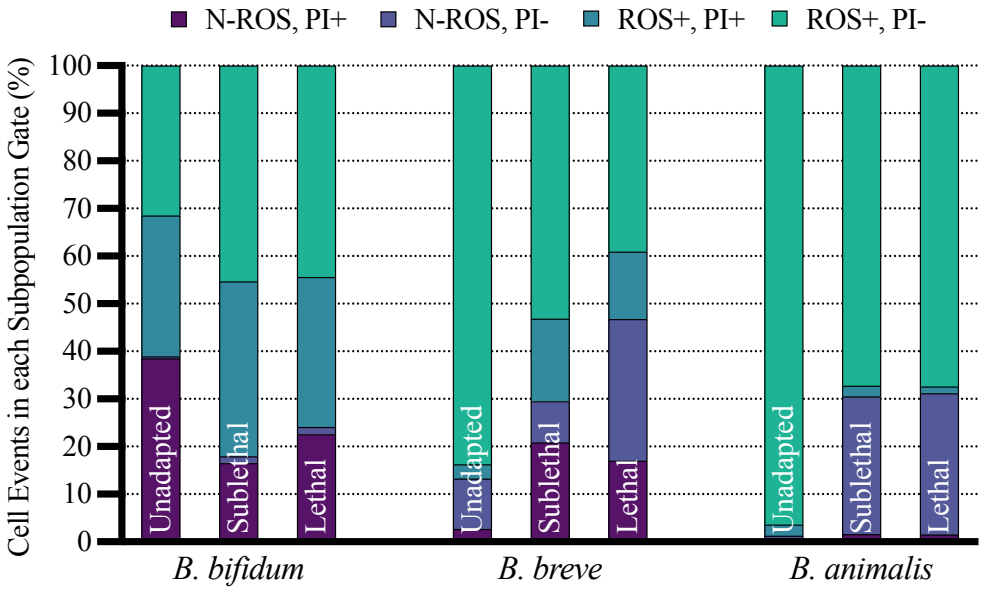


Figure 5.3 Relative proportions of unoxidised, membrane-damaged (N-ROS, PI+), unoxidised, membrane-intact (N-ROS, PI), oxidised, membrane-damaged (ROS+, PI+) and oxidised, membrane-intact (ROS+, PI-) subpopulations of unadapted, sublethal- and lethal H_2O_2-treated *Bifidobacterium* species following exposure to the lethal H_2O_2 challenge (1 mM H_2O_2; 30 min).

Overall, the lethal H_2O_2-treated cells demonstrated the highest amount of cells in an unoxidised state and an intact membrane (N-ROS, PI-) compared to the same subpopulation of the unadapted and sublethal H_2O_2-treated cells within each *Bifidobacterium* species.

5.3.3 Stress response of stress-adapted *Bifidobacterium* spp.

Considering the results from the FC assays, the overall observations were that the extension of the adaptation treatment, through the addition of the lethal H_2O_2 treatment, resulted in variants exhibiting an enhanced response to the lethal challenge compared to both the unadapted and sublethal H_2O_2-treated cells. As a result, the subsequent sections involved the analysis of the lethal H_2O_2-treated (hereinafter referred to as 'stress-adapted') *Bifidobacterium* variants.

5.3.3.1 Growth kinetics of stress-adapted Bifidobacterium spp.

As shown in Table 5.1, *B. breve* exhibited the highest growth rate among the three species, irrespective of the H_2O_2 concentration and adaptation treatment ($P < 0.001$) (Table 5.1). Interestingly, the growth rates for *B. bifidum* and *B. animalis* were higher in the presence of 0.1 and 1 mM H_2O_2 than under anaerobic conditions ($P < 0.001$) (Tables 5.1 and 5.2).

Table 5.1 Analysis of variance showing the effect of bacterial species, stress adaptation and growth condition on the growth rate and the maximum population density of *Bifidobacterium* spp. ($n = 4$)

		***P*-Value**	
Source of Variation	**DF**	**Growth Rate**	**MPD[1]**
Main Effects			
Bifidobacterium species (*B. bifidum, B. breve, B. animalis*)	2	< 0.001	< 0.001
Adaptation Treatment (Unadapted, Adapted)	1	-	< 0.001
Growth Condition (0, 0.1, 1 mM H_2O_2)	2	< 0.001	< 0.001
Interactions			
Species x Growth Condition	4	< 0.001	< 0.001

[1]MPD = maximum population density, as indicated by the OD^{36h}.

Table 5.2 Growth rates of unadapted and stress-adapted *Bifidobacterium* spp. during 36 hours of incubation under 0, 0.1 and 1 mM H_2O_2.[1]

	Growth rate (OD units/h)					
Growth	***B. bifidum***		***B. breve***		***B. animalis***	
Condition	**Unadapted**	**Stress Adapted**	**Unadapted**	**Stress Adapted**	**Unadapted**	**Stress Adapted**
0 mM H_2O_2	$0.007^{Aa} \pm 0.009$	$0.072^{Ab} \pm 0.007$	$0.158^{Ac} \pm 0.003$	$0.148^{Ac} \pm 0.001$	$0.006^{Aa} \pm 0.001$	$0.040^{Aab} \pm 0.007$
0.1 mM H_2O_2	$0.142^{Ba} \pm 0.002$	$0.093^{Ab} \pm 0.013$	$0.162^{ABa} \pm 0.001$	$0.176^{Ba} \pm 0.003$	$0.085^{Bb} \pm 0.007$	$0.068^{Bb} \pm 0.036$
1 mM H_2O_2	$0.150^{Ba} \pm 0.003$	$0.149^{Ba} \pm 0.001$	$0.187^{Bb} \pm 0.011$	$0.178^{Bab} \pm 0.001$	$0.093^{Bc} \pm 0.002$	$0.043^{Ad} \pm 0.059$

[1]Values are mean ± standard deviation ($n = 4$).

Statistically significant differences ($P < 0.05$) between means are indicated by different small-caps letters within the same row, and different all-caps letters within the same column.

In particular, the growth rates for both unadapted and stress-adapted *B. bifidum* were several folds higher under 0.1 mM H_2O_2 and 1 mM H_2O_2 concentrations than under anaerobic conditions (0 mM H_2O_2) (0.007 ± 0.009 OD units/h and 0.072 ± 0.007 OD units/h, respectively) ($P < 0.05$) (Table 5.2).

Similarly, the MPD of *B. breve* was observed to be generally greater than *B. bifidum* and *B. animalis* ($P < 0.001$) (Tables 5.1 and 5.3). When considering the impact of the H_2O_2 concentrations, Table 5.3 shows that the MPDs for all *Bifidobacterium* spp. were higher under both 0.1 and 1 mM H_2O_2 concentrations than under anaerobic conditions ($P < 0.05$), particularly for *B. bifidum* and *B. breve* ($P < 0.05$) (Table 5.1). In contrast, *B. animalis* generally displayed a lower MPD than *B. bifidum* and *B. breve*, exhibiting similar MPDs regardless of the growth condition ($P < 0.05$) (Tables 5.1 and 5.3). In addition, an increase in the MPD was observed in the stress-adapted *Bifidobacterium* variants. However, this increase was significant ($P < 0.005$) only for *B. bifidum* and *B. animalis* under an anaerobic environment (Tables 5.1 and 5.3).

5.3.3.2 Morphology of stress-adapted Bifidobacterium spp. under lethal challenge

From Figure 5.4, it is clear that the morphological responses of *Bifidobacterium* spp. to the lethal oxidative stress were observed to be species-dependent. After exposure to the lethal challenge, distinct morphological changes were observed among the *Bifidobacterium* species. (Figure 5.4). Most significantly, both unadapted and stress-adapted *B. breve* cells displayed clear cell rupture and shrinkage, indicating complete cell envelope collapse, as seen in Figures 5.4 (B) and (E), respectively. However, stress-adapted *B. animalis* (Figure 5.4 (C)) displayed similar damage but to a lesser extent (Figure 5.4 (F)). The unadapted cells of *B. breve* developed larger cellular pores and significantly collapsed cell walls (Figure 5.4 (B)). Furthermore, in *B. bifidum*, there was an enhancement in EVs production (Figure 5.4 (A)), more so in the unadapted *B. bifidum* cells (Figure 5.4 (D)).

Table 5.3 MPD of unadapted and stress-adapted *Bifidobacterium* spp. during 36 hours of incubation under 0, 0.1 and 1 mM H_2O_2.[1]

	Maximum Population Density (OD_{36h})					
Growth	***B. bifidum***		***B. breve***		***B. animalis***	
Condition	**Unadapted**	**Stress Adapted**	**Unadapted**	**Stress Adapted**	**Unadapted**	**Stress Adapted**
0 mM H_2O_2	$0.142^{Aa} \pm 0.028$	$0.536^{Ab} \pm 0.245$	$0.789^{Ac} \pm 0.015$	$0.758^{Abc} \pm 0.044$	$0.249^{Aa} \pm 0.027$	$0.576^{Abc} \pm 0.041$
0.1 mM H_2O_2	$0.708^{Babd} \pm 0.014$	$0.632^{Aad} \pm 0.208$	$0.821^{Aac} \pm 0.013$	$0.946^{Bbc} \pm 0.046$	$0.370^{Ade} \pm 0.043$	$0.551^{Ae} \pm 0.055$
1 mM H_2O_2	$0.717^{Bae} \pm 0.016$	$0.729^{Aae} \pm 0.048$	$0.947^{Aac} \pm 0.012$	$1.145^{Bbc} \pm 0.353$	$0.317^{Ad} \pm 0.060$	$0.532^{Ade} \pm 0.038$

[1]Values are mean ± standard deviation ($n = 4$).

Statistically significant differences ($P < 0.05$) between means are indicated by different small-caps letters within the same row, and dif-ferent all-caps letters within the same column.

MPD = maximum population density, as indicated by the OD_{36h}.

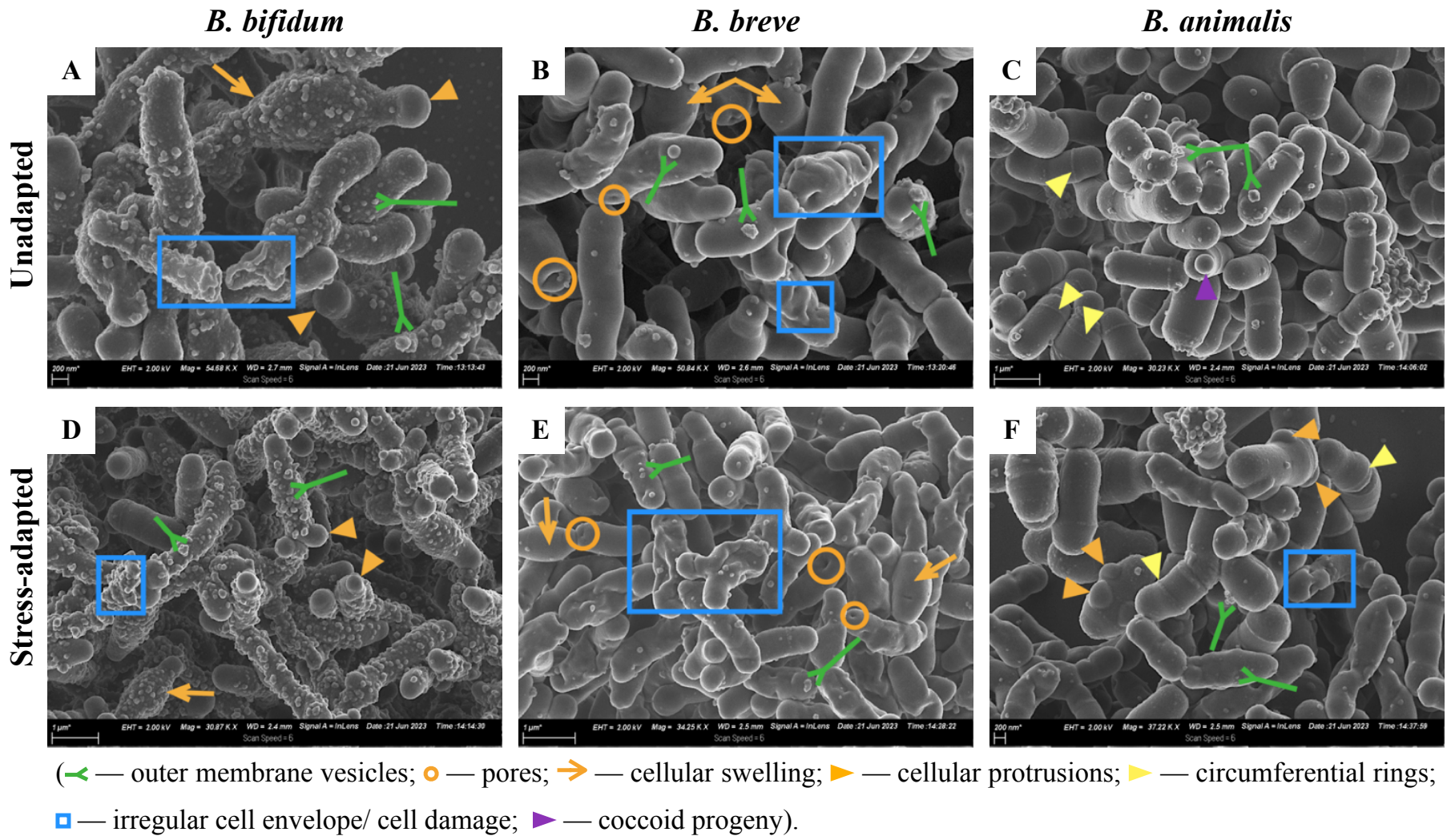


Figure 5.4 Scanning electron images of unadapted (A-C) and stress-adapted (D-F) *B. bifidum* (A and D), *B. breve* (B and E) and *B. animalis* (C and F) cells after exposure to lethal challenge (1 mM H_2O_2; 30 min).

Interestingly, stress-adapted *B. animalis* displayed more pronounced circumferential rings (Figure 5.4 (F)) after the lethal challenge. Additionally, the stress-adapted *B. bifidum* rods (Figure 5.4 (D)) were more elongated than the unadapted cells (Figure 5.4 (A)). In contrast, unadapted *B. animalis* cells appeared shorter and thicker (Figure 5.4 (C)). In *B. bifidum* and *B. breve*, both the unadapted (Figures 5.4 (A) and (B), respectively) and stress-adapted cells (Figures 5.4 (D) and (E), respectively) exhibited swelling towards coccoid ends.

5.3.3.3 Ultrastructure of stress-adapted Bifidobacterium spp. under lethal challenge

Figure 5.5 illustrates a distinct reorganisation of the internal structures of unadapted and stress-adapted *Bifidobacterium* spp. in response to the lethal challenge. Following exposure to the lethal H_2O_2 challenge, membrane ruptures were evident in both unadapted and stress-adapted *B. bifidum* cells (Figures 5.5 (A) and (D)), with several extracellular particles, possible remnants of the ruptured cell walls or vesicles (extracellular or cytoplasmic). In *B. breve*, thinning of the cell envelope was observed for both unadapted and stress-adapted cells (Figures 5.5 (B) and (E), respectively). Unadapted *Bifidobacterium* spp. exhibited disorganised cytoplasms, with blank patches within, indicative of condensed compartments concentrated in one area of the cell (Figures 5.5 (A), (B), and (C)). In particular, unadapted *B. breve* and *B. animalis* displayed a distinct disruption of their cytoplasmic homogeneity (Figures 5.5 (B) and (C), respectively). Furthermore, unadapted *B. breve* displayed distinctive ring-like structures with lucent contents of unknown function (Figure 5.5 (B)). Electron-dense particles were observed within the *B. breve* cells, possibly representing precipitated and aggregated organelles (Figures 5.5 (B) and (E)), with a more distinct observation in the stress-adapted cells (Figure 5.5 (E)). Cytoplasmic vesicles were notably present in unadapted *B. bifidum* and *B. animalis* (Figures 5.5 (A) and (C), respectively). Additionally, a significant increase in ribosomal occurrence was observed in *B. animalis* (Figures 5.5 (C) and (F)).

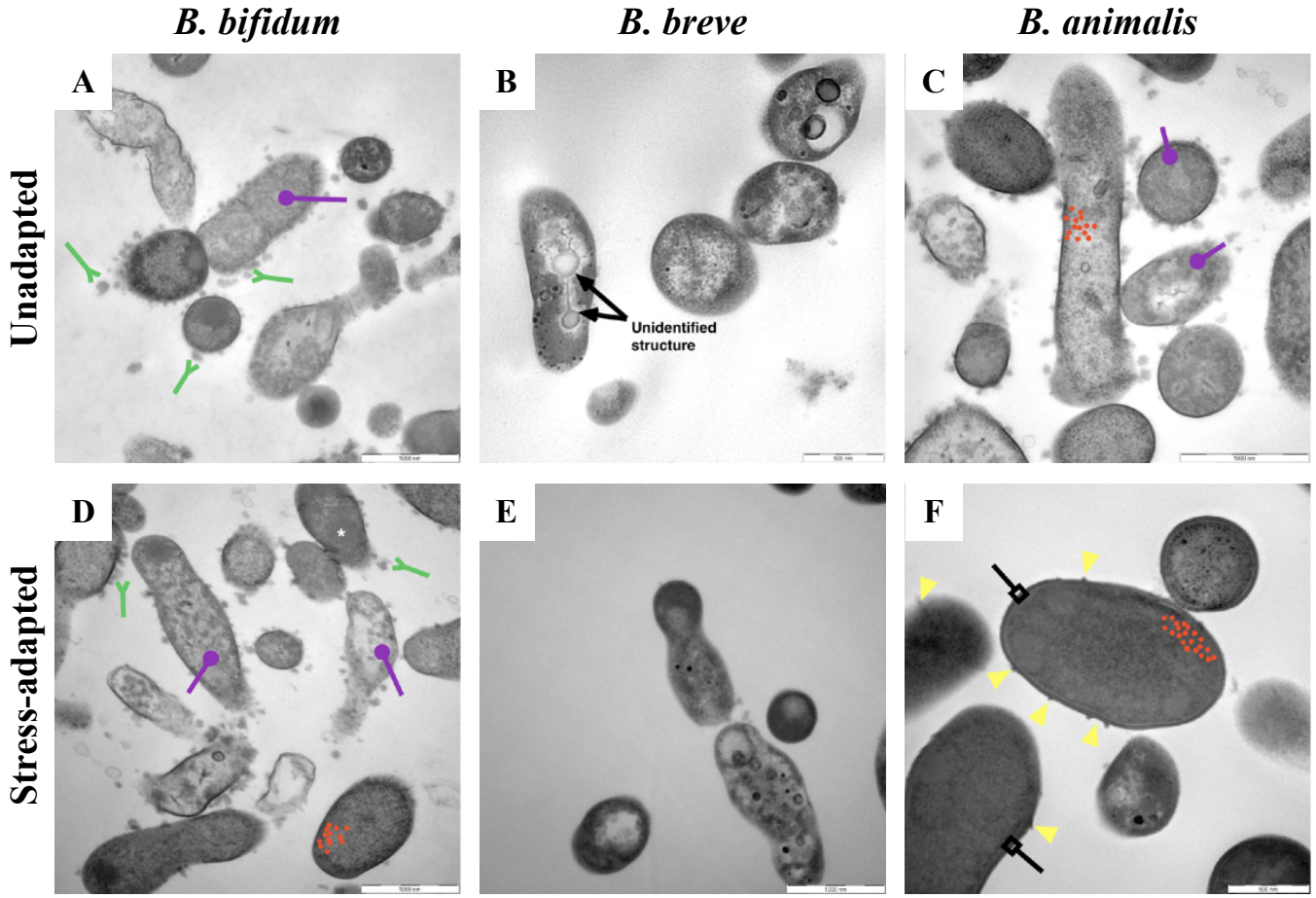


Figure 5.5 Transmission electron images of unadapted, control (A-F) and stress-adapted (G-L) *B. bifidum* (A, D, G, J), *B. breve* (B, E, H, K) and *B. animalis* (C, F, I, L) cells. Images represent respective bacterial species before (A-C and G-I) and after exposure (D-F and J-L) to lethal oxidative stress (1 mM H_2O_2; 30 min).

5.4 DISCUSSION

This study investigated the physiological responses of three *Bifidobacterium* species to the lethal H_2O_2 challenge, following stress adaptation treatments, i.e. sublethal- and subsequent sublethal- plus lethal H_2O_2 treatments.

5.4.1 H_2O_2-treated *Bifidobacterium* variants show improved oxidation state and membrane stability under lethal challenge

We observed variations in the oxidative stress responses across the three H_2O_2-treated *Bifidobacterium* species. Notably, the unadapted *B. bifidum* cells exhibited higher susceptibility to membrane damage due to the lethal challenge than the H_2O_2-treated cells. These results highlight the O_2-sensitive nature of *B. bifidum* (Kawasaki et al., 2018*a*). *B. bifidum* possibly does not possess strongly inducible enzyme systems to deal with the high H_2O_2 challenge used in this study. A previous study by Satoh et al. (2019) isolated and characterised an inducible TrxR from *B. bifidum* under aerobic conditions, an indication that some *B. bifidum* strains can adapt to oxidative stress. With the high H_2O_2 used in the experiment, it is possible that the AhpC-TrxR system of the unadapted *B. bifidum* cells was overloaded with H_2O_2, being unable to efficiently detoxify the damage-inducing H_2O_2 (Satoh et al., 2019).

In contrast, the H_2O_2-treated *B. bifidum* cells demonstrated better membrane integrity when faced with the lethal H_2O_2 challenge. Previous research has highlighted the capacity of *Bifidobacterium* spp. to alter its cell envelope composition under environmental stress (Oberg and Broadbent, 2016; Wei et al., 2019). In particular, the studies have identified that alterations in the cell membrane lipid composition of *Bifidobacterium* spp. are crucial for survival in stress conditions (Oberg and Broadbent, 2016; Wei et al., 2019). Thus, the H_2O_2 treatments potentially prepared *B. bifidum* cells to counteract the harmful effects of oxidative stress on the membrane, possibly by modifying the membrane's fluidity and composition or through repair mechanisms (Schöpping et al., 2022). However, no distinct patterns were discernible between the sublethal- and the lethal H_2O_2-treated cells for *B. bifidum*.

In contrast to *B. bifidum*, the unadapted *B. breve* cells demonstrated greater membrane integrity when faced with the lethal H_2O_2 challenge than the H_2O_2-treated cells. However, the lethal H_2O_2-treated *B. breve* cells were in a lower oxidation state than both the unadapted and the sublethal H_2O_2-treated cells. These findings indicate that the H_2O_2 treatments potentially prepared the cells for the lethal challenge at a biochemical level, particularly the lethal H_2O_2-treated *B. breve* cells, rather than enhancing membrane-associated response mechanisms. Zomer et al. (2009) observed an upregulation of genes associated with the AhpC/F system in *B. breve* UC2003 following exposure to oxidative stress. However, the researchers reported the SOS response to be activated upon DNA damage within the cell (Zomer et al., 2009). Therefore, the deduced hypothesis from these findings is that the oxidative stress response of *B. breve* could be triggered by damage to internal biomolecules, a process initiated by ROS passing through the cell membrane and to the intracellular space. Nonetheless, additional studies are required to validate this hypothesis.

Interestingly, *B. animalis* presented with the highest proportion of cells with intact membranes whilst in an oxidised state, and this was more evident in the unadapted cells. These findings suggest that *B. animalis* may inherently be well-equipped to handle oxidative stress, having a rapid response mechanism in place for such conditions (Schöpping et al., 2023). Furthermore, the results potentially indicate an intrinsic ability of *B. animalis* to modify its membrane composition as an inherent adaptation to oxidative stress (Oberg and Broadbent, 2016; Wei et al., 2019). Some studies have noted upregulation of genes encoding for long-chain fatty-acid-CoA ligase and cyclopropane-fatty acyl-phospholipid synthase in *Bifidobacterium* spp. in response to oxidative stress (Oberg et al., 2013; Zuo et al., 2018; Wei et al., 2019), which resulted in an increased content of short-chain and cyclopropane fatty acids in the cell membranes, thereby decreasing their membrane fluidity and offering enhanced resistance to oxidation and potential protection against oxidative damage (Grogan and Cronan, 1997; An et al., 2014). Therefore, *B. animalis* potentially responded to the lethal challenge by altering the membrane composition, displaying enhanced membrane integrity maintenance during the FC assay.

Furthermore, the stress-adapted *B. animalis* cells were in a lower oxidation state than the unadapted cells. These findings indicate a potential activation of H_2O_2 detoxification systems in stress-adapted *B. animalis* cells (Kawasaki et al., 2018*a*; Schöpping et al., 2023). Moreover, the lethal H_2O_2 treatment resulted in a much lower CRG fluorescence, indicating an

enhanced detoxification potential (Aakko et al., 2014; Oberg and Broadbent, 2016). In line with these findings, Schöpping et al. (2023) reported elevated transcription levels of oxidative stress-associated genes in *B. animalis* subsp. *lactis* BB-12 that were likely indicative of a rapid response to oxidative stress. Generally, *B. animalis* is known for its intrinsic aerotolerance which is much higher than the other *Bifidobacterium* spp. included in this study (Kawasaki et al., 2018*a*). The inherent genetic advantage is due to inducible enzymes that can detoxify ROS, such as AhpC and TrxR (Zuo et al., 2014). Both AhpC and TrxR are components of the prokaryotic alkyl hydroperoxide reductase enzyme systems that detoxify H_2O_2 by reducing it to alcohol and water (Zuo et al., 2014). In the system, AhpC functions as a peroxidase, while TrxR acts as a reductase (Zuo et al., 2014; Schöpping et al., 2022). These results suggest that, as an inherently aerotolerant species, the natural defence system against H_2O_2 not only contributes to the protection of the cells from oxidative damage but, also plays a role in protecting other structural components of the cell. In addition, while the sublethal H_2O_2 treatment bolstered the cells' capacity to neutralise ROS and also reinforced their membrane structures against oxidative damage (Schöpping et al., 2022), the lethal H_2O_2 treatment further augmented these capabilities, enabling the *B. animalis* cells to remain less oxidised and maintain intact cell membranes even under the lethal challenge.

The membrane integrity and oxidation states of the unadapted and H_2O_2-treated *Bifidobacterium* spp. after exposure to the lethal H_2O_2 challenge showed some variation among the species. This potentially indicates a species-dependent variation in the susceptibility to oxidative stress among the *Bifidobacterium* species. Similar observations were made by Oberg et al. (2012), who reported significant differences in the cell membrane fatty acid composition of two nearly genetically similar strains of *B. lactis*. These differences were also found to considerably impact each species' intrinsic oxidative stress resistance (Oberg et al., 2012). Thus, stress adaptation itself is a characteristic specific to species or even individual strains, which involves alterations in the lipid composition of the membrane (Alvarez-Ordóñez et al., 2008; du Toit et al., 2013). Evidently, these findings call for species-tailored stress adaptation procedures, as there was a clear species-dependent response to oxidative stress in terms of membrane integrity retention and ROS detoxification.

5.4.2 Growth kinetics reflects species-specific responses to oxidative stress

The study also aimed to assess whether adaptation to oxidative stress influenced the growth kinetics of *Bifidobacterium* spp. when exposed to either anaerobic conditions or previously sublethal and lethal H_2O_2 concentrations of 0.1 mM and 1 mM, respectively.

The results from the growth kinetics analyses revealed that *B. breve* growth remained consistent regardless of both the adaptation and the varying levels of H_2O_2 concentrations. Contrary to the expected stress responses of bacteria, which include energy conservation leading to reduced growth rates and smaller cell sizes under stress conditions (Novitsky and Morita, 1976; Gilbert et al., 1990), *B. breve* did not exhibit these common stress responses. This outcome suggests a unique resilience mechanism in *B. breve*, allowing it to maintain steady growth under conditions that typically challenge other bacterial strains.

Interestingly, both *B. bifidum* and *B. animalis* exhibited a positive correlation between growth rate and H_2O_2 concentration, as well as between the MPD and H_2O_2 concentration. Likewise, the stress adaptation led to increased MPDs for *Bifidobacterium* spp. under varying H_2O_2 concentrations. The reason for this could be a potential increased hydrophobicity and autoaggregation of the cells in order to physically retard and exclude H_2O_2 and ROS from entering the cells or causing damage (Zuo et al., 2018; Schöpping et al., 2023). After exposing *B. longum* BBMN68 to 3% oxygen, Zuo et al. (2018) observed an increase in cell surface hydrophobicity and autoaggregation. The researchers speculated that these changes might function to reduce oxygen penetration into the cells, suggesting a protective adaptation against oxidative stress (Zuo et al., 2018). This suggests that similar mechanisms might be at play in these species, including *B. breve*, enhancing their resilience to oxidative stress through the physical exclusion of H_2O_2 and ROS, thereby contributing to their improved growth and population density under the H_2O_2 concentrations in this study.

5.4.3 Stress adaptation enhanced morphological modifications in *Bifidobacterium* spp.

The production and release of EVs were consistently expressed by *B. bifidum* irrespective of adaptation, which resulted in a textured cell surface. Notably, these vesicles appeared larger

and more frequent following lethal challenge, hinting at their role in the *B. bifidum*'s stress response. In line with these findings, EVs released by Gram-positive bacteria have been classified as containing fatty acids, phospholipids, peptidoglycan, cytoplasmic proteins, and membrane-associated molecular chaperones (Bose et al., 2020). The rough cell surface observed might reflect not just EV release but also disrupted cell division, a possible negative effect of oxidative stress (Ahn et al., 2001). Furthermore, Qian et al. (2011) observed the formation of intracellular granules in *Bifidobacterium* spp. grown in MRS without a reducing agent, suggesting that the development of vesicle-like structures is integral to the stress response mechanism of *Bifidobacterium* species. Conversely, *B. breve* and *B. animalis* only displayed EV formation after the lethal challenge, although to a lesser extent in the stress-adapted cells. Therefore, the observed patterns in EV production and surface morphology among *B. bifidum*, *B. breve*, and *B. animalis* in response to oxidative stress highlight the diverse adaptive strategies of *Bifidobacterium* species.

Upon exposure to the lethal challenge, the cells, with the exception of *B. animalis*, underwent elongation. This observation is in agreement with the literature, whereby *Bifidobacterium* spp. can change their morphology from rod-shaped to more energy-conserved coccoid shape when faced with environmental stress (Ultee et al., 2019). Similarly, *B. bifidum* species altered their morphology during adaptation to bile stress, tending towards a smaller and more uniform size (Margolles et al., 2003). The cellular elongation observed for *B. bifidum* and *B. breve* cells after the lethal challenge might be attributed to filamentation (Ultee et al., 2019).

5.4.4 Exposure to the lethal H_2O_2 challenge resulted in ultrastructural adaptations in *Bifidobacterium* spp.

The findings from the TEM analysis revealed distinct ruptured membranes in unadapted and stress-adapted *B. bifidum* cells after lethal challenge and resonates with the findings in the SEM analysis. However, these structural observations in *B. bifidum* closely mirror the findings reported by Kim et al. (2018), particularly in the context of viable but non-culturable (VBNC) cells of *E. coli*. Similarities such as membrane irregularities, electron-lucent areas in the cytoplasm, and particles near the inner membrane indicate the potential transition of *B. bifidum* cells towards VBNC cells. Furthermore, similar structural observations were made in unadapted

Bifidobacterium cells in this study. In alignment with this, Amor et al. (2002) determined a significant portion of cells that presented as metabolically active during FC measurements were unable to be cultivated during plate counts. Furthermore, *Bifidobacterium* spp. has been proven to enter a VBNC state during nutrient depletion, thereby remaining metabolically active but unculturable (Lahtinen et al., 2008). These findings provide insight into a possible survival strategy employed by *Bifidobacterium* spp. under harsh conditions, where cells might transition into a VBNC state.

In addition, the thinning of the cell envelope of *B. breve* potentially had to do with a loss in EPSs following exposure to the lethal challenge. Although this observation might be unexpected, as EPSs are often associated with antioxidant activity (Li et al., 2014), this might not have been the case for *B. breve*. However, similar observations were made in *B. longum* subsp. *longum* BBMN68 during bile stress adaptation (Jiang et al., 2016). Resonating with the findings during the FC analysis, indicating that *B. breve* may employ alternative stress response mechanisms not detectable in this study.

5.5 CONCLUSION

Overall, sublethal and lethal H_2O_2 treatments of *Bifidobacterium* spp. improve membrane integrity retention and oxidative stress detoxification upon re-exposure to oxidative stress. As an inherently O_2-sensitive bacterium, unadapted *B. bifidum* is significantly vulnerable to H_2O_2 exposure. However, adaptation enhances its membrane-associated responses, potentially including alterations in membrane fluidity and composition. In contrast, H_2O_2 treatment of *B. breve* strengthens its internal oxidative stress responses, notably through the reduction of ROS, thereby improving the cell's internal redox state when confronted with oxidative stress. *B. animalis* naturally exhibits enhanced tolerance to oxidative stress, characterised by its dual-stress response of preserving membrane integrity and effectively managing intracellular oxidation levels. Compared to the other two bifidobacteria, *B. animalis* shows a superior and more efficient response to oxidative stress, as reflected in growth kinetics analysis. Moreover, extending the adaptation treatment with the addition of lethal H_2O_2 further enhances the stress responses of the species compared to those that only received the sublethal treatment.

However, the adaptation of *B. breve* does not affect its growth kinetics in the presence of H_2O_2, indicating a unique resilience mechanism that supports steady growth under typically challenging conditions. Meanwhile, *B. animalis* exhibits consistent growth rates and MPD across different conditions, aligning with its oxygen-tolerant nature. Furthermore, the adaptation treatment enhances several morphological and intracellular responses to oxidative stress. Notably, *B. bifidum* shows enhanced production and release of EVs due to the adaptation treatment, pointing towards a critical role in the stress response. Additionally, adaptation prompts unique structural changes in *B. breve* and *B. animalis*. Notably, the morphological adaptations observed, such as cellular elongation and the formation of unique cellular structures, underscore the distinct responses of each *Bifidobacterium* species. However, this study's findings underscore the species-specific responses to oxidative stress and, in turn, their species-specific responsiveness to stress adaptation.

CHAPTER 6

Yoghurt shelf-life study of stress-adapted *Bifidobacterium* spp.

This study aimed to evaluate the effect of oxidative stress adaptation of *Bifidobacterium* spp. on their viability, interactions with yoghurt starter cultures, and physicochemical characteristics (i.e. pH, TA, ORP, syneresis) during fermentation and storage, to sustain an appropriate therapeutic dose (6 log CFU/g viable cells) of the probiotic throughout the product's lifetime, whilst ensuring a chemically stable yoghurt product. Adapted variants of *B. bifidum*, *B. breve*, and *B. animalis* subsp. *animalis* were isolated following H_2O_2 treatments, while unadapted *Bifidobacterium* counterparts served as the control. Following the addition of the *Bifidobacterium* cultures, the yoghurt was fermented to pH 4.6 at 37 °C. Bacterial viability was evaluated using a novel propidium monoazide-quantitative polymerase chain reaction (PMAxx-qPCR) method alongside standard plate count methods, while physicochemical characteristics were monitored weekly over a 28-day shelf-life study at 4 °C. The adapted *Bifidobacterium* strains exhibited improved regulation of acid production, which may enhance their survival and contribute to the chemical stability of the yoghurt. This adaptation potentially conferred cross-protection, enabling these bacteria to utilise nutrients more efficiently and manage metabolic responses during fermentation. While adapted *B. bifidum* and *B. breve* demonstrated improved viability and metabolic advantages, *B. animalis* displayed inherent resilience regardless of adaptation. However, the PMAxx-qPCR assay suggested that *Bifidobacterium* spp. declines in culturability yet remain viable during yoghurt storage rather than decline in cell numbers. This poses contradicting evidence concerning the viability of *Bifidobacterium* spp. in yoghurt. Additionally, the study highlighted the accuracy and reliability of the PMAxx-qPCR method in quantifying bacterial species in mixed-species yoghurt, providing insights into their viability even in the viable but non-culturable (VBNC) state. These findings suggest that adaptation treatments can enhance probiotic viability, potentially extending shelf-life and improving the health benefits of yoghurt, which are in line with regulatory standards.

Keywords: *Bifidobacterium* species, probiotic viability, yoghurt, stress adaptation, propidium monoazide, quantitative-PCR

6.1 INTRODUCTION

Probiotics are defined as 'live microorganisms that confer a health benefit to the host when administered in sufficient quantities' (FAO/WHO, 2002; Hill et al., 2014). Adding probiotic cultures into functional foods has become increasingly popular among food manufacturers, responding to a growing consumer trend towards foods that offer health benefits. Dairy products, particularly yoghurt, are distinguished for their efficacy as carrier foods for delivering probiotics (Zhang et al., 2023*b*). Sufficient quantities of viable microorganisms are a requirement for any therapeutic benefit to the consumer. Hence, it is advised that probiotic foods should have approximately 6 log CFU per gram at the time of consumption (Neffe-Skocińska et al., 2018), where the typical serving size in South Africa for yoghurt is 200 grams, the minimum level of viable probiotic cells should be 8 log CFU per serving. Thus, the amount of probiotics added to yoghurt should aim to meet or surpass this minimum level to ensure effectiveness (Sibanda et al., 2024). Among the probiotic bacterial genera and species, *Bifidobacterium* spp. are considered to be the most beneficial with an extensive range of health benefits linked to their inclusion in foods (IDF and Federation, 2015; Nyanzi et al., 2021; Sibanda et al., 2024).

However, the incorporation of *Bifidobacterium* spp. into probiotic foods like yoghurt is a significant challenge. As anaerobes, *Bifidobacterium* spp. are generally sensitive to oxygen, rendering them vulnerable to oxidative stress encountered in the yoghurt manufacturing process (Kawasaki et al., 2018*a*; Schöpping et al., 2022; Sibanda et al., 2024). Currently, only a selection of species and strains are commercially available for producing probiotic yoghurt, such as the moderately aerotolerant *B. animalis* subsp. *lactis* (He et al., 2023). Their oxidative stress susceptibility often leads to a failure to maintain their viability above the recommended therapeutic levels throughout the product's shelf-life. Consequently, the inability to sustain viability in foods limits their potential to provide adequate health benefits to consumers upon intake.

Stress adaptation refers to the process through which a microorganism that is inherently susceptible to stress, acquires the capability to withstand such conditions. It can be used to enhance the tolerance of *Bifidobacterium* spp. to oxidative stress and, therefore, improve their viability during yoghurt production and storage (Schöpping et al., 2022; Sibanda et al., 2024). This approach entails exposing cells to sublethal stress conditions to induce physiological and genetic

responses that enable an organism to cope with the stress (Settachaimongkon et al., 2015). The induced stress response can prepare the cells for future stressful environments, improving their chances of survival (Schöpping et al., 2022). Subsequently, culturing of the pre-exposed cells in a lethal stress environment across several generations can solidify the stress response, resulting in variants that consistently exhibit improved tolerance to stress. Limited research exists on the molecular basis of such adaptive responses (Oberg et al., 2013; Jin et al., 2015; Wei et al., 2019). There is scant research on the application and survival of stress-adapted *Bifidobacterium* spp. within food products, particularly yoghurt. Therefore, this study aimed to investigate the effect of oxidative stress adaptation of three probiotic *Bifidobacterium* species, i.e. *B. bifidum*, *B. breve* and *B. animalis* subsp. *animalis*, on their survival in yoghurt during fermentation and refrigerated storage, to sustain an appropriate therapeutic dose (6 log CFU/g viable cells) of *Bifidobacterium* cells throughout yoghurt fermentation and storage. Additionally, this study aimed to evaluate the accuracy and applicability of a novel PMAxx-PCR method developed by Marole et al. (2024) for selectively quantifying viable starter and *Bifidobacterium* cultures in mixed-species probiotic yoghurt compared to the traditional plate counting technique.

6.2 MATERIALS AND METHODS

6.2.1 Yoghurt starter and probiotic cultures

The stress-adapted variants of *B. bifidum*, *B. breve* and *B. animalis* used in this study, were prepared as described in Chapter 5. This study used the unadapted wild types of these cultures as a control.

6.2.2 Preparation of probiotic cultures for yoghurt fermentation

Unadapted and stress-adapted *Bifidobacterium* spp. stock cultures were resuscitated in MRS-C broth, incubating at 37 °C for 48 h under anaerobic conditions. A 1% inoculation of the resuscitated cultures was made into 50 ml MRS-C and incubated anaerobically at 37°C for 48 h.

Before inoculating the yoghurt milk preparation, the subcultured broth was centrifuged at 5000 × g for 10 min, and the cell pellet was resuspended in 10 ml of pasteurised milk.

6.2.3 Yoghurt preparation, fermentation and cold storage

Raw cow's milk (3% fat, 8% fat-free solids, 11% total solids) was obtained from the University of Pretoria Experimental Farm (Pretoria, South Africa). The milk was mixed with 1.5% (w/v) stabiliser (acetylated distarch adipate, E1422) in sterilised Schott bottles, each containing 250 ml of milk, and allowed to hydrate for 30 minutes at 4 °C before pasteurisation at 90 °C for 10 minutes. The milk was cooled to 37 °C and inoculated with the yoghurt starter culture (LYOFAST Y 259 A, SACCO, Como, Italy) and the respective probiotic preparations, as depicted in Table 6.1. The *Bifidobacterium* spp. were added to the milk preparations at 6 to 7 log CFU/ml before fermentation. The yoghurt fermentation was carried out at 37 °C. The pH was monitored, as stated below, every 30 minutes until it decreased to pH 4.6 in approximately 3.5 h. After fermentation, the yoghurts were cooled to 4 °C and stored for 28 days. Bacteriological and physicochemical analyses were performed at 7-day intervals for the 28 days of the yoghurt shelf-life (0, 7, 14, 21 and 28 days). Duplicate yoghurt samples were prepared for each fermentation.

Table 6.1 Descriptive labels of the yoghurt preparations and their respective symbols.

	Culture added to yoghurt mix		**Culture added to yoghurt mix**
YBU –	Unadapted *B. bifidum*	YBA –	Stress-adapted *B. bifidum*
YRU –	Unadapted *B. breve*	YRA –	Stress-adapted *B. breve*
YAU –	Unadapted *B. animalis*	YAA –	Stress-adapted *B. animalis*

6.2.4 Physicochemical analysis of yoghurt during storage

6.2.4.1 Measurement of pH

The pH of each treatment was determined using a pH electrode for dairy products (HANNA® Instruments Inc., USA). The pH was measured at the start of fermentation, during each hour of fermentation, and every seven days of the shelf-life (0, 7, 14, 21 and 28 days).

6.2.4.2 Measurement of titratable acidity (TA)

During the shelf-life, the TA of each yoghurt sample was measured on days 0, 7, 14, 21, and 28. Each titration was carried out in triplicate. Twenty millilitres of diluted yoghurt sample (1⁄10 dilution in ddH_2O) was titrated with 0.1 N NaOH to a faint pink colour, using one drop of phenolphthalein as the indicator. The percentage of lactic acid in each probiotic yoghurt was calculated using the following equation:

$$\%\ \text{Lactic acid} = \frac{\text{titre} \times N \times 90}{M_x \times 10},$$

where the titre is the volume of 0.1 N NaOH used in the titration, N is the nomality of 0.1 N NaOH, and $M_\times$ is the initial mass of the yoghurt before diluted.

6.2.4.3 Measurement of oxidation-reduction potential

The yoghurt samples' oxidation-reduction potential (ORP) of the yoghurt samples was measured on days 0, 7, 14, 21, and 28 of the self-life study using an ORP meter (HANNA® Instruments Inc., USA). A duplicate measurement was taken within five seconds of the first measurement. Subsequently, the magnitude of change in ORP was calculated on days 7, 14, 21 and 28, using the following equation:

$$\Delta\text{ORP} = \text{ORP}_x - \text{ORP}_0,$$

where ORP_x is equal to the yoghurt ORP measurement on the day of analysis (day 7, 14, 21 or 28) and ORP_0 is equal to the yoghurt ORP measurement on Day 0 of the storage period.

6.2.4.4 Measurement of syneresis

A 10 g sample of yoghurt was placed in a 10 ml centrifuge tube and spun at 3500 rpm for 10 minutes and the syneresis was calculated as follows:

$$\% \text{ Syneresis} = \frac{\text{whey separated (g)}}{\text{initial yoghurt mass (g)}} \times 100,$$

6.2.5 Determination of starter and probiotic culture viability during storage

The yoghurt samples were plated in duplicate on appropriate selective media according to Table 6.2. The M17 (*S. thermophilus*) plates were incubated at 37 °C for 24 hours. The MRS-NNLP (*Bifidobacterium* spp.) and MRS (pH 5.4) (*L. bulgaricus*) plates were incubated anaerobically at 37 °C for 48 h in anaerobic jars made anoxic by anaerogen sachets (AnaeroGen™, Oxoid Ltd, Basingstoke, UK). The viability proportion index (VPI) of the probiotics at the end of the yoghurt storage period (day 28) was calculated using the following equation:

$$VPI = \frac{\text{final cell population (cfu/ml)}}{\text{initial cell population (cfu/ml)}}$$

6.2.6 Bacterial quantification with propidium monoazide-quantitative polymerase chain reaction (PMAxx-qPCR)

Each bacterial species in the yoghurt was quantified with PMAxx-qPCR (Marole et al., 2024) on days 0 and 28 of the shelf-life. The VPI for the relevant bacterial cultures were calculated as described previously (Section 6.2.5).

Table 6.2 Selective media composition for the enumeration of bacterial species from probiotic yoghurt.

Bacterial species	Media	Reference
S. thermophilus	M17 agar supplemented with 1% lactose (v/w)	Shah (2000)
L. bulgaricus	MRS agar adjusted to pH 5.4 with 0.13% (v/v) acetic acid	
Bifidobacterium spp.	MRS agar supplemented with nalidixic acid sodium salt (0.015 g/l), neomycin sulfate (0.001 g/l), lithium chloride (3 g/l), paromomycin sulfate (0.2 g/l), L-cysteine (5 g/l) (MRS-NNLP Agar)	

6.2.6.1 Bacterial cell extraction

Yoghurts containing unadapted- and stress-adapted *Bifidobacterium* spp. (3.0 g), were adjusted to pH 6.3 by adding of 1 M NaOH, followed by digestion with 1 M tri-sodium citrate (3.0 ml) (García-Cayuela et al., 2009). The resulting cell pellets were isolated by centrifuging at 10 000 $\times$ g for 10 min at 4 °C before washing thrice with PBS (pH 7.3) (Yang et al., 2021). The cell pellets were resuspended in 400 μl ultra-pure H_2O or MRS broth before PMAxx-treatment.

6.2.6.2 PMAxx-treatment

The propidium monoazide (PMAxx)-treatment was performed on the extracted bacterial cell suspensions, adding 2 μl of PMAxx™ dye (Biotium Inc., Hayward, CA, USA) solution (20 mM) to the cell suspensions to achieve a final concentration of 100 μM. Subsequently, the samples were cooled on ice, and exposed to a 500 W halogen light source for 15 min, with the samples periodically rotated. After PMAxx treatment, the samples were centrifuged at 6 000 $\times$g for 10 min and the cell pellets were used to extract DNA.

6.2.6.3 DNA extraction

Genomic DNA was extracted from 100 μl of PMAxx-treated cells using the ZR DNA Miniprep Kit (Zymo Research, USA). The extracted DNA was quantified using a Qubit™ 4 Fluorome-

ter with the dsDNA High Sensitivity (HS) assay kit (Invitrogen™, Thermo Fisher Scientific, Waltham, USA). Before quantitative polymerase chain reaction (qPCR) analysis, the DNA concentration was adjusted to 5 ng/μl with PCR-grade ultra-pure water. Standardised DNA extracts were stored at -20 °C until qPCR analysis.

6.2.6.4 Real time qPCR (RT-qPCR)

The quantitative polymerase chain reaction (qPCR) reaction was prepared to a final volume of 10 μl. It contained 5.0 μl of 2 $\times$ TB Green R© Advantage R© qPCR Premix (TB Green dye, full-length Taq DNA Polymerase, hot-start antibody, dNTPs, and buffer) (Takara Bio Inc, Mountain View, CA, USA), 0.2 μl of the forward primer (10 μM), 0.2 μl of the reverse primer (10 μM), 1.0 μl of the template DNA (0.5 ng) and 3.6 μl of nuclease-free water. The primers used in the qPCR assays were as reported by Marole et al. (2024) and are given in Table 6.3.

The qPCR conditions involved denaturation at 96 °C for 30 s for all species, followed by 35 cycles of a second denaturation at 95 °C for 5 s. Annealing occurred at 62 °C for 20 s for *S. thermophilus* and *L. bulgaricus*, followed by extension at 72 °C for 20 s and 6 s for *S. thermophilus* and *L. bulgaricus*, respectively. Annealing and extension were carried out simultaneously for *Bifidobacterium* spp. at 60 °C for 20 s. A melt curve analysis was performed in the range of 45 to 96 °C at increments of 0.5 °C.

Table 6.3 Species-specific primers for q-PCR of probiotics and yoghurt cultures.

Organism		**Primer sequence**	**References**
S. thermophilus	F	5′ -CGTGGTGTTGTTCGTGTTAATGA-3′	Fan et al. (2021)
	R	5′ -CGGCAATACCTTCATCAAGTTGT-3′	
L. delbrueckii	F	5′ -AGACTCTTGACTTGGGTGAAGC-3′	Marole et al. (2024)
	R	5′ -GTTCTGTGGGTCTTGATTGAGC-3′	
Bifidobacterium spp.	F	5′ -AAGCCGTTCCTGATGCCTATC-3′	Marole et al. (2024)
	R	5′ -GAGGTAACGGTGGTGGTCTG-3′	

F: Forward Primer; R: Reverse Primer

A no-template control (NTC) (1.0 μl nuclease-free water as the template) was included during each run. Each sample was analysed in duplicate. Quantitative polymerase chain reaction (qPCR) reactions were conducted on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and cycle threshold (Ct) values were automatically determined using a single threshold mode, identifying the point where the threshold intersected with background levels and the point of onset of the exponential phase of the qPCR reaction.

6.2.6.5 Construction of standard curves

The standard curves for *B. animalis* were created using the method described by Ilha et al. (2016). Cultures of *B. animalis* were resuscitated in MRS-C broth at 37 °C for 48 h before centrifugation at 10000 × g for 10 min at 4 °C. The resulting cell pellet was washed twice with PBS (pH 7.3) and resuspended in 400 μl MRS broth. The pure culture was stained with PMAxx and the genomic DNA was extracted, as described in Section 6.2.6.3. The genomic DNA isolated was then 10-fold serially diluted in PCR-grade ultra-pure water.

The number of bacterial DNA copies was calculated based on the genome size of *B. animalis* ATCC® 25527™ (1 932 963 bp) (Loquasto et al., 2011), using Avogadro's constant (6.022 × 1023), the amount of DNA, the average molecular weight of double-stranded-DNA (660 Da) per base pair, and a conversion factor of 1 × 109 to convert to nanogram (ng) (scienceprimer.com). Furthermore, the genomic DNA was serially diluted 10-fold in PCR-grade ultra-pure water up to the final genome copy number, and qPCR reactions were performed, as described in Section 6.2.6.4. Additionally, PMAxx-treated cell suspensions of *B. animalis* were serially diluted 10-fold and spread-plated (100 μl) on MRS-C agar before incubating anaerobically at 37 °C for 48 h.

Following analysis, standard curves for *B. animalis* were generated by plotting the respective Ct values versus the logarithm of the DNA copy number. The linear dynamic range (LDR), slope (S), and correlation coefficient (R^2) were determined. Additionally, the qPCR amplification efficiency (E) was calculated using the following equation (Broeders et al., 2014):

$$E = 100 \times \left(10^{-1/S} - 1\right),$$

The respective Ct values were plotted versus the logarithm CFU/ml of *B. animalis* to determine the limit of quantification (LOQ).

6.2.6.6 Calculation of true log CFU/g from standard curves

The standard curves created by Marole et al. (2024) for *S. thermophilus*, *L. bulgaricus*, *B. bifidum*, and *B. breve* were used to calculate the true log CFU/g from the respective Ct values obtained during qPCR. For *B. animalis*, the standard curve constructed in this study was used. From the Ct values obtained during qPCR, the true log CFU per gram of yoghurt was calculated using the following equation (Ilha et al., 2016):

$$\textit{Bacterial count (log CFU/g)} = \log_{10}\left(\frac{A \times B \times C}{D \times E}\right),$$

where the bacterial count is in log CFU/g, A is the antilog of the CFU/g of yoghurt obtained from the Ct value of the DNA extracted from the yoghurt sample using the equation from the standard curve (Ct vs. log CFU/g), B is the DNA concentration of the DNA extracted from the yoghurt sample, C is the volume of the DNA extract (400 μl), D is the standardised DNA concentration (0.5 ng/μl), and E is the initial yoghurt weight (3.0 g).

6.2.7 Statistical analysis

ANOVA at $\alpha = 0.05$ was used to test for any significant differences in probiotic viability and the physicochemical properties of yoghurt samples with adapted and unadapted *Bifidobacterium* spp. over the shelf-life period. Least Squares Means were adjusted where applicable. Analysis was done using SPSS Version 29 software (IBM, USA) and GraphPad Prism 10.0 software. All experiments were replicated. Furthermore, a PCA was conducted using GraphPad Prism 10.0 software.

6.3 RESULTS

6.3.1 Fermentation kinetics of yoghurt containing unadapted and stress-adapted *Bifidobacterium* species

Figure 6.1 illustrates the progression of pH levels during the fermentation process of yoghurt containing unadapted and adapted *Bifidobacterium* species. Alongside the pH profiles, the viability of the probiotic species at the beginning and end of fermentation is presented in Figure 6.2.

All the milk samples exhibited a pH of approximately 6.5 at the start of fermentation (Figure 6.1). Regardless of the adaptation treatment or the *Bifidobacterium* spp., all samples reached a pH of 4.6 within 3.5 hours of fermentation. Despite this overall similarity in the fermentation curves, some differences were observed in the acidification between yoghurts prepared with adapted *Bifidobacterium* spp. compared to unadapted ($P < 0.001$).

Table 6.4 Analysis of variance showing the effect of bacterial species and adaptation treatment on the pH and viability of unadapted or adapted *Bifidobacterium* spp. viability of probiotic yoghurt during fermentation (0 - 3.5 h).

		***P*-Value**	
Source of Variation	**DF**	**pH**	**Probiotic Viability**
Main Effects			
Bifidobacterium spp. (*B. bifidum, B. breve, B. animalis*)	2	< 0.001	< 0.001
Stress Adaptation (Unadapted, Adapted)	1	-	< 0.001
Fermentation Time (Probiotic Viability) (0, 3.5 h)	1	-	< 0.001
Interactions			
Spp. x Stress Adaptation	2	-	< 0.001
Spp. x Fermentation Time (Probiotic Viability)	2	-	< 0.001
Stress Adaptation x Fermentation Time (Probiotic Viability)	1	-	< 0.001
Spp. x Stress Adaptation x Fermentation Time (Probiotic Viability)	2	-	< 0.001

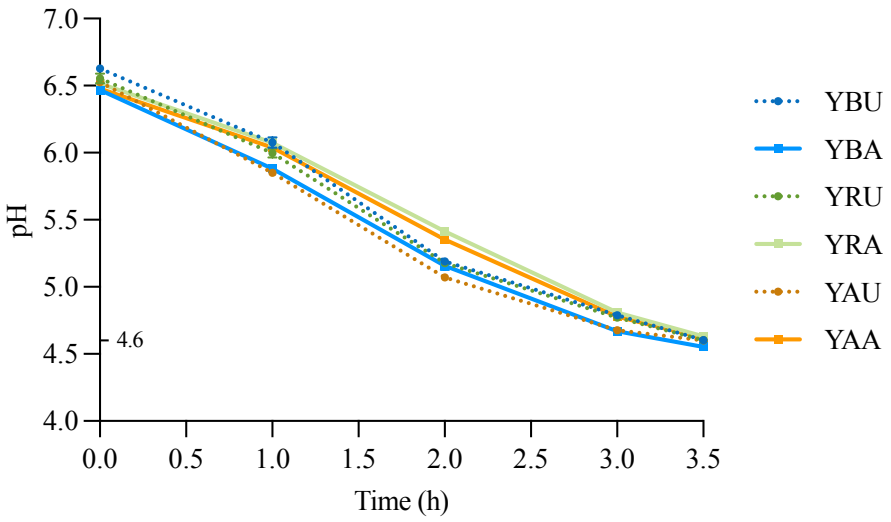


Figure 6.1 Effect of stress adaptation on the pH (left) and *Bifidobacterium* spp. viability (right) during fermentation of yoghurt containing (A) *B. bifidum*, (B) *B. breve*, (C) *B. animalis*. Values are the means ± standard deviation ($n = 4$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

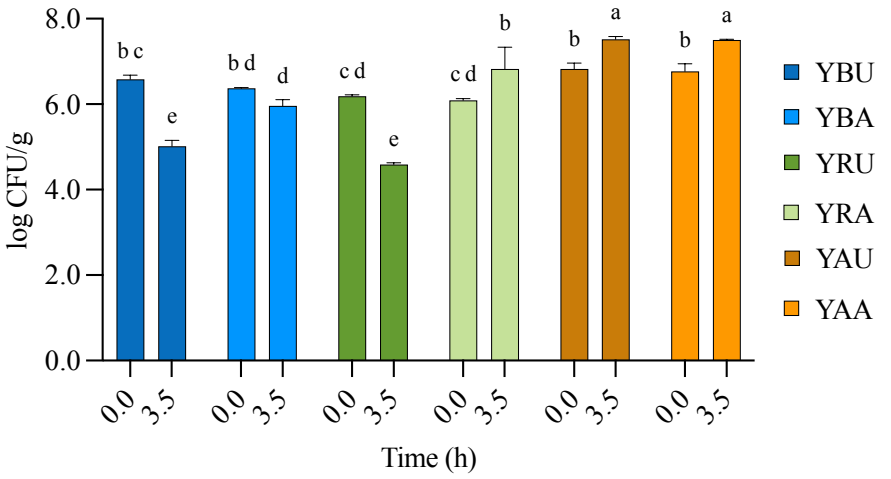


Figure 6.2 Effect of stress adaptation on the *Bifidobacterium* spp. viability during fermentation of yoghurt containing *B. bifidum*, *B. breve* and *B. animalis*. Values are the means ± standard deviation ($n = 4$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Yoghurts containing adapted *Bifidobacterium* spp. (YBA, YRA and YAA) demonstrated a more consistent and gradual decline in pH after the first hour of fermentation, with a noticeable slowdown in the rate of pH decline after the third hour of fermentation (Figure 6.1).

The yoghurt preparations were inoculated with the respective *Bifidobacterium* spp. between 6 - 7 log CFU/g before fermentation (Figure 6.2). Furthermore, in Figure 6.2, the different *Bifidobacterium* species and their adapted counterparts exhibited varied responses to the fermentation process. For instance, while the viability of *B. bifidum* decreased during fermentation, the adapted counterpart demonstrated enhanced viability retention, whereby 1 log difference was observed between the unadapted and adapted *B. bifidum* post-fermentation ($P < 0.0001$) (Figure 6.2).

Furthermore, it is evident in Figure 6.2 that the adaptation of *B. breve* resulted in greater viability retention and an increase in bacterial counts during fermentation ($P < 0.0001$). Therefore, the fermentation process itself favoured the growth of the adapted *B. breve* (Figure 6.2). Conversely, the opposite effect of the fermentation process was observed for unadapted *B. breve* in the yoghurt, whereby its viability declined from 6.2 log CFU/g to 4.6 log CFU/g by the end of fermentation (Figure 6.2).

Table 6.5 Analysis of variance showing the effect of bacterial species and adaptation treatment on the physicochemical characteristics (pH, TA, ΔORP, Syneresis) of yoghurt containing either unadapted or adapted *Bifidobacterium* spp., during 28 days of refrigerated storage.

		P-Value		
Source of Variation	**DF**	**pH**	**TA**	**ΔORP**
Main Effects				
Bifidobacterium spp. (*B. bifidum, B. breve, B. animalis*)	2	< 0.001	-	< 0.001
Stress Adaptation (Unadapted, Adapted)	1	0.011	< 0.001	< 0.001
Shelf-life (pH, TA, Syneresis) (0, 7, 14, 21, 28 days)	4	< 0.001	< 0.001	-
Shelf-life (ΔORP) (7, 14, 21, 28 days)	3	-	-	< 0.001

6.3.2 Shelf-life study of yoghurt with unadapted and stress-adapted *Bifidobacterium* species

6.3.2.1 Physicochemical characteristics of yoghurt fermented with unadapted and stress-adapted Bifidobacterium species

During the shelf-life, a decrease in pH was observed across all yoghurt samples over the 28-day storage period, with an initial pH of approximately 4.6 (Figure 6.3). The most pronounced decline in pH for all yoghurts occurred within the first seven days of storage ($P < 0.001$). Specifically, the pH declined from 4.6 to approximately 4.4 in all yoghurt samples by day 7 (Figure 6.3). Overall, there were no observable differences between yoghurt samples based on *Bifidobacterium* spp. and adaptation treatment. At the end of the shelf-life period, the pH levels across all yoghurt samples converged to approximately 4.3 or lower (Figure 6.3).

Throughout storage, yoghurts with unadapted *Bifidobacterium* spp. showed higher lactic acid percentages compared to yoghurt with the adapted counterparts ($P < 0.05$), as seen in Figure 6.3. This was particularly pronounced on day 14 for all three *Bifidobacterium* yoghurts ($P < 0.001$) and extended to day 28 specifically for *B. bifidum* and *B. breve* ($P < 0.001$) (Figure 6.3). Notably, at the end of shelf-life, unadapted *B. bifidum* and *B. breve* yoghurts (YBU and YRU) exhibited 1.5% lactic acid, distinctly higher than the yoghurt with their adapted counterparts (YBA and YRA) ($P < 0.001$), which presented with 1.2% lactic acid (Figures 6.3 (A) and (B)).

Table 6.6 shows the change in ORP of the yoghurt samples over the 28-day cold storage. Overall, there was a notable increase in ORP across all the yoghurt samples during the shelf-life ($P < 0.001$). Interestingly, the peak increase in ORP was observed after day 21, whereby yoghurt samples containing unadapted and adapted *B. bifidum* (YBU and YBA, respectively) showed increases of 170 mV and 194 mV from day 0, respectively. Differences in the magnitude of change in ORP during storage between yoghurt samples containing different *Bifidobacterium* spp. were observed ($P < 0.001$). Distinctly, yoghurts prepared with *B. animalis* (YAU and YAA) demonstrated slower ORP increases compared to yoghurts prepared with *B. bifidum* (YBU and YBA) and *B. breve* (YRU and YRA). A notable difference in the change in ORP was observed between yoghurt samples containing unadapted or adapted *Bifidobacterium* species ($P < 0.001$).

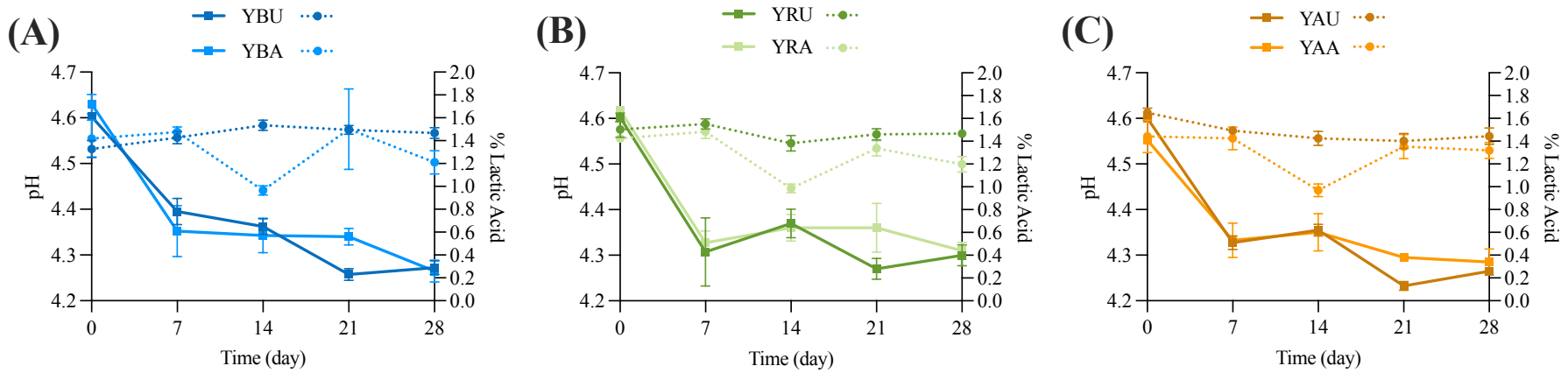


Figure 6.3 Effect of stress adaptation on the pH (left) and titratable acidity (% lactic acid) (right) over the 28-day cold storage (4 °C) period of yoghurt containing (A) *B. bifidum*, (B) *B. breve*, (C) *B. animalis*. Values are the means ± standard deviation ($n = 4$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Table 6.6 Change in ORP (ΔmV) over the 28-day cold storage (4 °C) period of yoghurt containing unadapted or adapted *Bifidobacterium* species.

Time	**Change in ORP (ΔmV)**					
(day)	**YBU**	**YBA**	**YRU**	**YRA**	**YAU**	**YAA**
0 - 7	128.4 ± 23.9	140.8 ± 21.4	154.1 ± 5.7	79.9 ± 5.6	114.7 ± 2.8	49.9 ± 9.9
0 - 14	147.0 ± 25.6	160.7 ± 3.8	169.1 ± 6.9	110.4 ± 4.8	130.3 ± 1.3	80.3 ± 4.7
0 - 21	169.9 ± 25.5	171.8 ± 19.7	194.0 ± 6.9	120.6 ± 2.1	154.8 ± 1.2	97.5 ± 6.1
0 - 28	152.8 ± 26.1	168.8 ± 17.7	180.1 ± 7.1	120.2 ± 2.2	146.8 ± 3.2	100.6 ± 5.8

Values are the means ± standard deviation (n = 4).

YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*;

YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*;

YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Table 6.7 Effect of stress adaptation on the syneresis (%) over the 28-day cold storage (4 °C) period of yoghurt containing *B. bifidum, B. breve*, and *B. animalis*.

Time	**% Syneresis**					
(day)	**YBU**	**YBA**	**YRU**	**YRA**	**YAU**	**YAA**
0	34 ± 1.3	33 ± 3.1	32 ± 4.3	38 ± 1.2	37 ± 1.7	36 ± 2.0
7	34 ± 2.9	38 ± 9.5	30 ± 5.1	37 ± 5.3	32 ± 4.5	41 ± 2.6
14	34 ± 2.3	39 ± 4.8	35 ± 1.8	37 ± 3.4	32 ± 3.1	40 ± 0.8
21	33 ± 2.0	41 ± 3.2	34 ± 3.9	38 ± 3.5	36 ± 4.8	42 ± 2.8
28	36 ± 1.4	42 ± 1.5	39 ± 3.5	34 ± 12.0	39 ± 4.2	46 ± 1.6

Values are the means ± standard deviation ($n = 4$).

YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Yoghurt samples with adapted *B. bifidum* and *B. breve* (YBA and YRA, respectively) exhibited consistently greater ORP changes throughout storage compared to their unadapted counterparts (YBU and YRU) ($P < 0.0001$). Conversely, yoghurt with adapted *B. animalis* (YAA) demonstrated slower ORP increases compared to the unadapted counterpart (YAU) ($P < 0.0001$).

Table 6.7 illustrates the progression of syneresis in all yoghurt samples throughout the storage period, with initial syneresis rates for yoghurts containing either unadapted or adapted *Bifidobacterium* spp. ranging from 32 to 38%. The particular *Bifidobacterium* species did not influence the syneresis of the yoghurt during storage. Additionally, the adaptation of *Bifidobacterium* spp. did not affect the yoghurt's syneresis during storage.

6.3.2.2 Viability of starter cultures, unadapted and stress-adapted Bifidobacterium spp. during yoghurt storage

The unadapted and adapted *Bifidobacterium* spp. on the viability of the yoghurt starter cultures was minimal. The viability of *S. thermophilus* remained at consistently high levels across all yoghurt samples, regardless of the presence of unadapted or adapted *Bifidobacterium* spp., with no deviation from initial counts of approximately 9.0 to 10.4 log CFU/g (data not shown). *L. bulgaricus* also maintained relatively stable viability during storage across all yoghurt samples, albeit starting from lower viability levels (between 3.7 and 4.7 log CFU/g) (data not shown). However, in yoghurt containing adapted *B. breve*, *L. bulgaricus* exhibited enhanced viability after fermentation, reaching 5.8 log CFU/g and maintaining this level throughout storage (data not shown).

The viability of unadapted and adapted *B. bifidum*, *B. breve*, and *B. animalis* in yoghurt over 28 days of storage are presented in Figure 6.4. As shown in the figure, *B. animalis* was the only species that maintained its viability above 6 log CFU/g throughout the shelf-life, with *B. bifidum* and *B. breve* experiencing a decline (Figure 6.4). The decline in *B. bifidum* and *B. breve* viability was also evident from the VPI results in Table 6.9.

Table 6.8 Analysis of variance showing the effect of bacterial species and adaptation treatment on the viability of unadapted or adapted *Bifidobacterium* spp. and resulting VPI[1], as measured by MRS-NNLP, in yoghurt during 28 days of refrigerated storage.

		***P*-Value**	
Source of Variation	**DF**	**Plate Counts**	**VPI_{28}**
Main Effects			
Bifidobacterium spp. (*B. bifidum, B. breve, B. animalis*)	2	< 0.001	< 0.001
Stress Adaptation (Unadapted, Adapted)	1	< 0.001	-
Shelf-life (0, 7, 14, 21, 28 days)	4	< 0.001	-
Interactions			
Spp. x Stress Adaptation	2	< 0.001	-
Spp. x Shelf-life	8	< 0.001	-
Stress Adaptation x Shelf-life	4	< 0.001	-
Spp. x Stress Adaptation x Shelf-life	8	< 0.001	-

[1]Viability Proportion Index

The adaptation treatment generally enhanced the viability of *B. bifidum* and *B. breve*, particularly notable on days 0, 21, and 28 ($P < 0.0001$), starting at higher initial viabilities post-fermentation (6.0 log CFU/g and 6.8 log CFU/g, respectively), compared to their unadapted counterparts (5.0 log CFU/g 4.6 log CFU/g, respectively) ($P < 0.0001$) (Figure 6.4). However, despite this initial advantage, adapted *B. bifidum* and *B. breve* exhibited a drop within the first 7 days of storage, contrasting with the more gradual decline observed for the unadapted counterparts in the first 21 days of storage ($P < 0.0001$) (Figure 6.4). In contrast, *B. animalis* exhibited an increase in viability from day 0 of storage to 8.9 log CFU/g for unadapted and 7.8 log CFU/g for adapted by day 28 (Figure 6.4). The increase in *B. animalis* viability is evident in Table 6.9, whereby the unadapted and adapted *B. animalis* VPI in the yoghurts were 1.18 and 1.05, respectively, during storage, indicating an increase over the 28 days.

The overall distinguishing characteristics of the yoghurt samples incorporated with stress-adapted and unadapted *Bifidobacterium* spp. were visualised through a PCA based on the viability levels and physicochemical properties of the yoghurt samples during 28 days of storage. As seen in Figure 6.5, the variables were reduced to two principal components (PC1 and PC2), accounting for 59.86% of the total variability of the data.

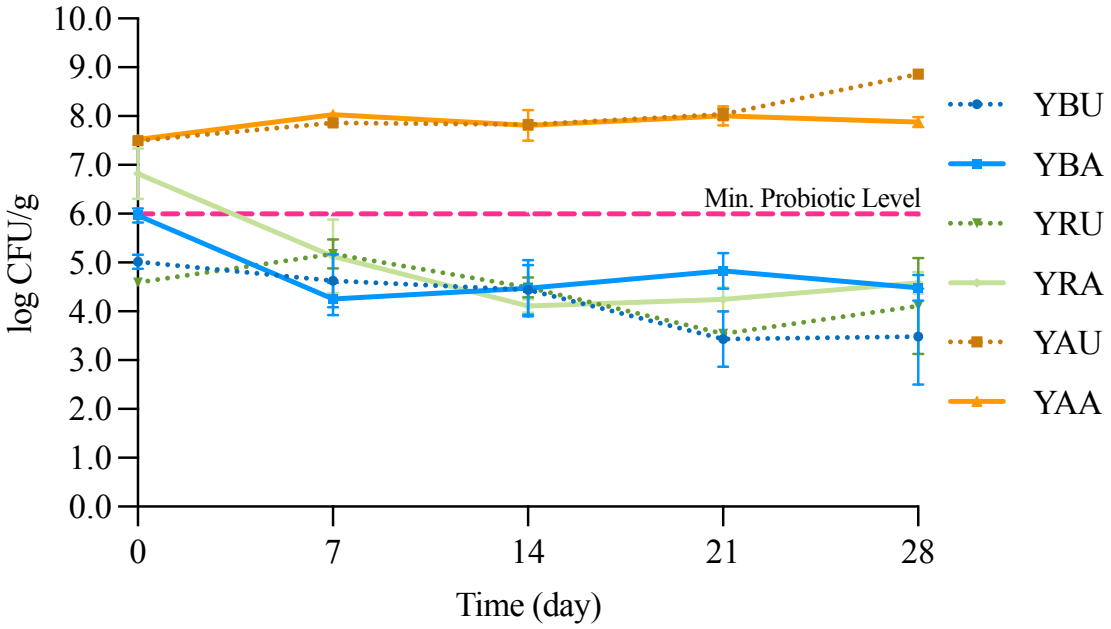


Figure 6.4 Effect of stress adaptation on probiotic viability in yoghurt containing (A) *B. bifidum*, (B) *B. breve*, and (C) *B. animalis* over the 28-day cold storage (4 °C) period. Values are the means ± standard deviation ($n = 4$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Table 6.9 The effect of stress adaptation on the viability proportion index (VPI) of *Bifidobacterium* spp. in yoghurt at the end of storage (day 28), as determined by MRS-NNLP.

Species	***B. bifidum***	***B. breve***	***B. animalis***
Unadapted	0.698 ± 0.211	0.895 ± 0.213	1.182 ± 0.019
Stress-adapted	0.752 ± 0.050	0.675 ± 0.062	1.047 ± 0.012

Values are the means ± standard deviation ($n = 4$).

A distinction between the yoghurt samples at 0 and at 7, 14, 21 and 28 days was apparent, with the main distinguishing characteristic being the pH that decreased in all samples as the days of storage increased. Furthermore, the PCA indicated a positive correlation between ORP and storage days while the same variables were negatively correlated with pH. While there were no discernible characteristics of yoghurt samples with stress-adapted and unadapted *Bifidobacterium* spp., the samples with *B. animalis* (YAA and YAU) had a high *Bifidobacterium* viability even as the TA increased.

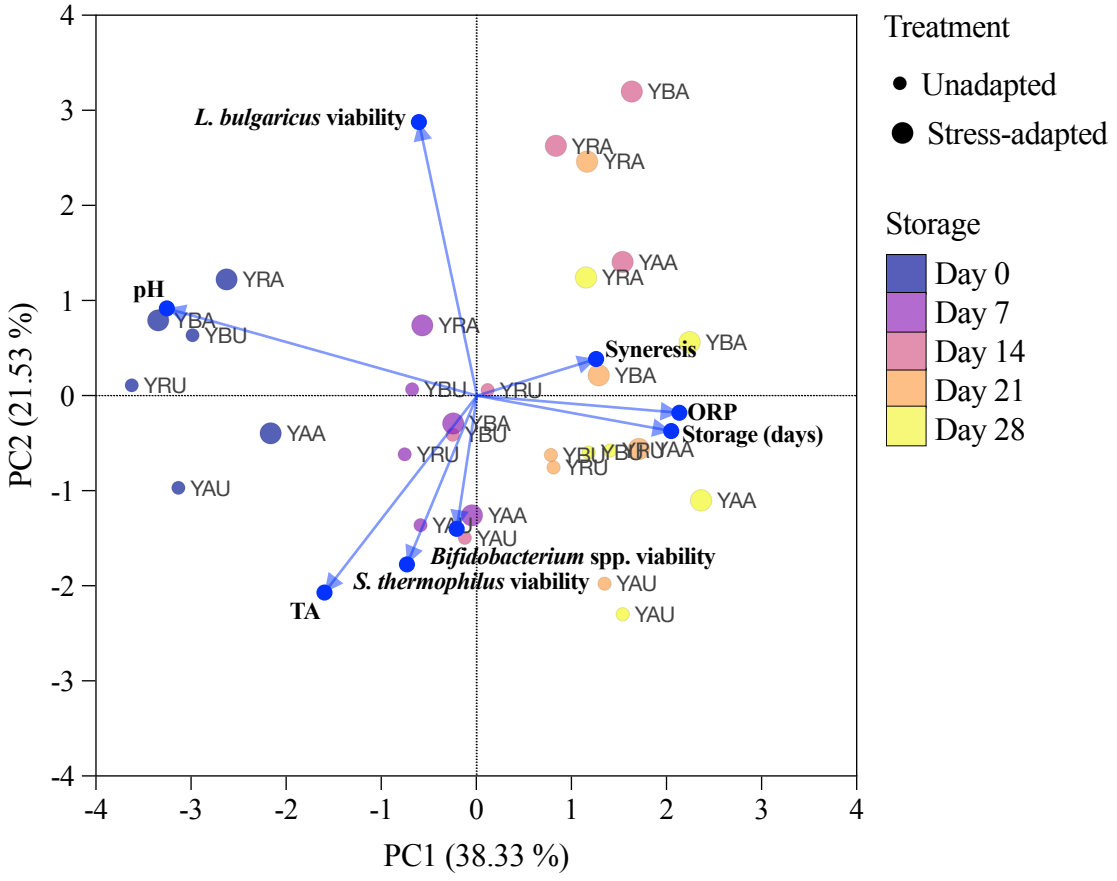


Figure 6.5 Principal component analysis (PCA) of physicochemical characteristics (pH, titratable acidity (TA), oxidation-reduction potential (ORP)) and viability of yoghurt bacteria (*S. thermophilus* and *L. bulgaricus*) and unadapted or stress-adapted *Bifidobacterium* spp. in yoghurt during refrigerated storage (Days 0, 7, 14, 21 and 28; 4 °C). Percentage of variance of PC1 and PC2 are indicated in parentheses. Arrows indicate the contribution of each variable to PC1 and PC2. YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

6.3.3 PMAxx-qPCR method of bacterial quantification in a mixed-species yoghurt as an alternative method to standard plate counting

6.3.3.1 Verification of primer specificity and standard curve of B. animalis subsp. animalis

The melt curve analysis, depicted in Figure 6.6, showed a clear peak at approximately 88 °C, indicating that the primers only amplified the target sequence, reinforcing the primer's reliability. The Ct values were used to construct the standard curve for *B. animalis* (Figure 6.7). The assay demonstrated a robust LDR from 10 to 10^6 genome copies for *B. animalis* (Figure 6.7). Furthermore, based on the standard curve in Figure 6.7, LOQ for *B. animalis* was established at 2 log CFU/g.

Subsequently, the Ct values were converted to log CFU/g using the standard curve, allowing for the enumeration of *B. animalis* during the PMAxx-qPCR assay. Furthermore, the standard curves of Marole et al. (2024) were used for the determination of *S. thermophilus*, *L. bulgaricus*, *B. bifidum* and *B. breve* viability during the PMAxx-qPCR.

6.3.3.2 Comparison of PMAxx-qPCR method to standard plate count method

A strong positive correlation (Pearson correlation coefficient of 0.9621) between bacterial counts obtained through the PMAxx-qPCR method and those determined by the standard plate counts (SPC) method was observed ($P < 0.0001$) (Figure 6.8 (A)). However, viability counts measured by the PMAxx-qPCR assay consistently exceeded those obtained via plate counting (Figure 6.8 (B)). The observed discrepancy between the enumeration methods averaged 27%, with variations ranging from -6% to 59% (Figure 6.8 (B)).

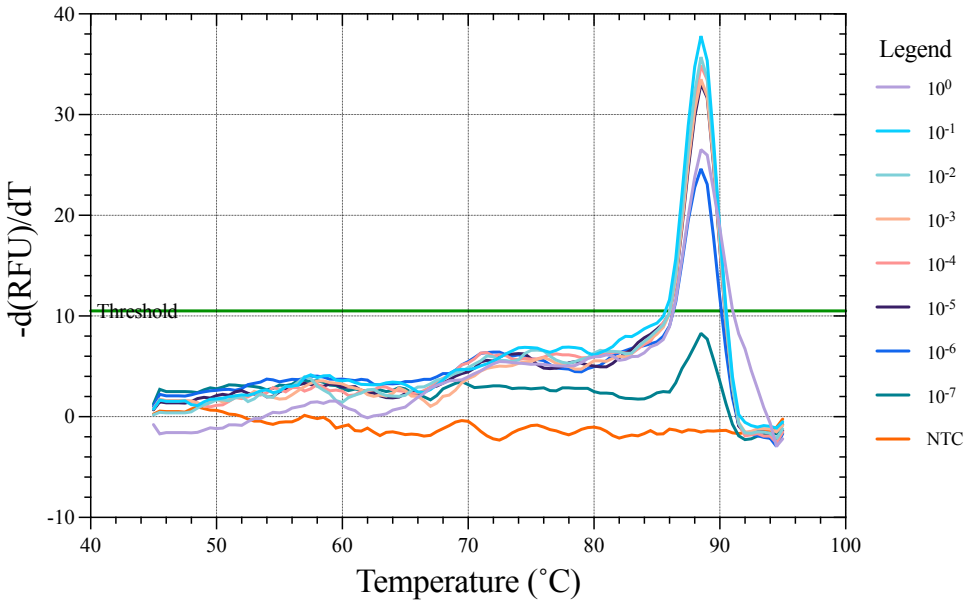


Figure 6.6 Melt curves showing the specificity of the primer for *Bifidobacterium* spp. against a serial dilution (100 - 10^{-7}) of the DNA of PMAxx-treated *B. animalis* subsp. *animalis* ATCC 25527 cells.

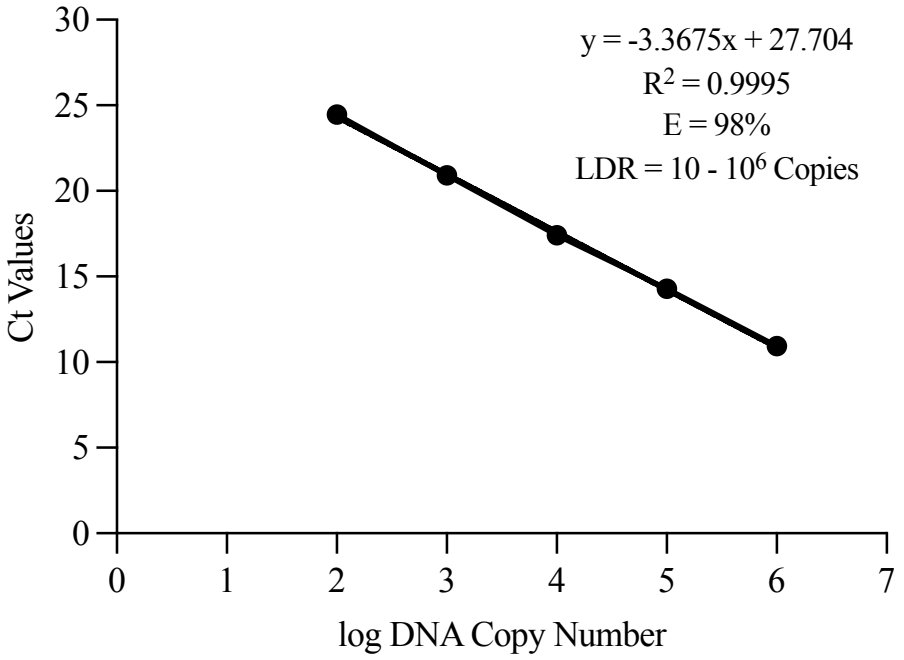


Figure 6.7 Standard curve of PMAxx-qPCR assay created and used for determining linear dynamic range (LDR), efficiency (E), and slope (K) for *B. animalis* subsp. *animalis* ATCC 25527. Each point represents the mean ± standard deviation of CT values ($n = 2$).

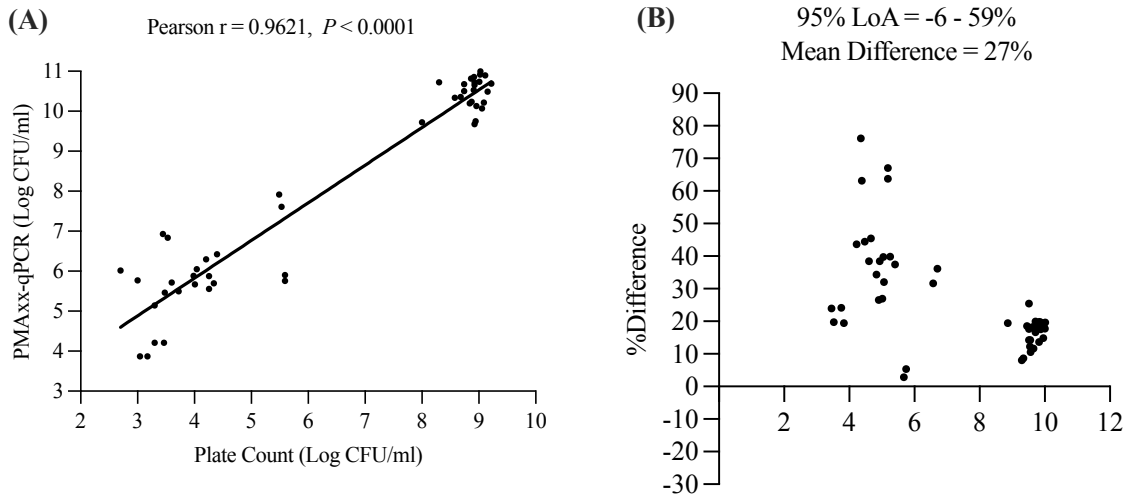


Figure 6.8 (A) Simple linear regression and (B) Bland-Altman method of comparison (% Difference vs. Average) of the PMAxx-qPCR method compared to the standard plate count method ($n = 48$). The Bland-Altman comparison = expressed as a percentage relative difference [100 × (PMAxx-qPCR count – Plate count)/average] vs. average. LoA: Limits of Agreement.

6.3.3.3 Starter cultures, unadapted and stress-adapted Bifidobacterium spp. viability in yoghurt before and after storage

The PMAxx-qPCR counts of *S. thermophilus* and *L. bulgaricus* were found to be considerably higher than those obtained from standard plate counts (Figure 6.9). Specifically, *S. thermophilus* levels remained stable between 10.0 and 11.0 log CFU/g throughout storage, regardless of the presence of either unadapted or adapted *Bifidobacterium* spp. (Figure 6.9 (A)), and were approximately 1 log higher than standard plate counts.

In contrast, while the viability of *L. bulgaricus* measured by PMAxx-qPCR was relatively stable during yoghurt storage, there were noticeable instances of influences of the presence of adapted *Bifidobacterium* species (Figure 6.9 (B)). Notably, *L. bulgaricus* in yoghurt containing adapted *B. bifidum* (YBA) and *B. breve* (YRA) saw increases in viability, i.e. 6.9 and 7.8 log CFU/g, respectively, by the end of storage, with the increase in being more pronounced in yoghurt with adapted *B. breve* ($P < 0.001$) (Figure 6.9 (B)). Conversely, the presence of *B. animalis* negatively affected *L. bulgaricus* viability, irrespective of the adaptation treatment (YAU and YAA), leading to a decrease to approximately 4.1 log CFU/g by day 28 ($P < 0.05$) (Figure 6.9 (B)).

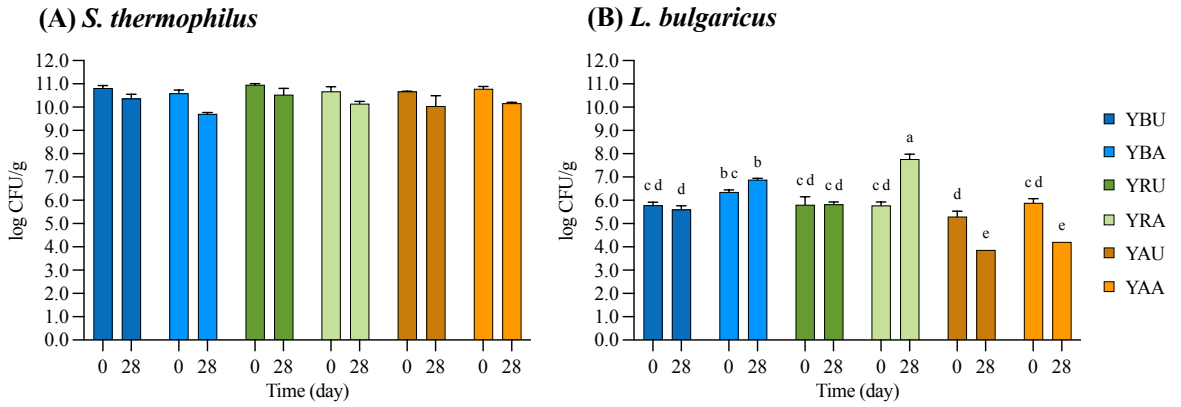


Figure 6.9 Viability of *S. thermophilus* (A) and *L. bulgaricus* (B) in yoghurt prepared with unadapted or stress-adapted *B. bifidum*, *B. breve* or *B. animalis*, on day 0 and 28 of refrigerated storage (4 °C), as determined by PMAxx-qPCR. Values are the means ± standard deviation ($n = 2$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Table 6.10 Analysis of variance showing the effect of bacterial species and adaptation treatment on the viability of unadapted or adapted *Bifidobacterium* spp. and resulting VPI[1], as measured by PMAxx-qPCR, in yoghurt during 28 days of refrigerated storage.

		***P*-Value**	
Source of Variation	**DF**	**PMAxx-qPCR**	**VPI_{28}**
Main Effects			
Bifidobacterium spp. (*B. bifidum, B. breve, B. animalis*)	2	< 0.001	< 0.001
Stress Adaptation (Unadapted, Adapted)	1	< 0.001	-
Interactions			
Spp. x Stress Adaptation	2	< 0.001	-
Spp. x Shelf-life	2	0.013	-

The PMAxx-qPCR results suggested no impact of adaptation on *Bifidobacterium* spp. viability in the yoghurt over time, albeit revealing species-specific differences. Notably, *B. breve* exhibited the highest viability in the yoghurt throughout storage, regardless of stress adaptation, consistently maintaining viability levels between 9.7 and 10.0 log CFU/g (Figure 6.10). Furthermore, both unadapted and adapted *B. breve* were stable during storage, demonstrated by the highest VPI values among the three *Bifidobacterium* spp. in the yoghurt, i.e. 0.97 and 0.95 (Table 6.11).

In comparison, both unadapted and adapted *B. bifidum* started with similar viability levels around 8.0 log CFU/g, decreasing significantly to 6.5 log CFU/g by day 28, marking the most substantial decline among the species ($P < 0.0001$) (Figure 6.10), which was reflected by the lowest VPI of approximately 0.81 (Table 6.11). *B. animalis* showed a minimal reduction in viability, starting from initial viabilities of about 7.0 log CFU/g and ending slightly lower, indicating its greater stability with VPIs of 0.90 and 0.94 for unadapted and adapted strains, respectively (Figure 6.10 and Table 6.11). Furthermore, the PMAxx-qPCR assay revealed that all three *Bifidobacterium* species remained above the recommended therapeutic minimum probiotic levels in yoghurt throughout storage (Figure 6.10).

Table 6.12 presents the estimated shelf-life of yoghurts prepared with either unadapted or adapted *Bifidobacterium* species. This estimation is based on the standard plate counts, and the PMAxx-qPCR counts of the *Bifidobacterium* spp. observed on days 0, 7, 14, 21, and 28 of storage (Figures 6.4 and 6.10), alongside the adherence to the recommended minimum probiotic concentration in yoghurt of 6 log CFU/g (FAO/WHO, 2003). Considering this criterion, the standard plate counts indicated that the adaptation of *Bifidobacterium* spp. improved the shelf-life of probiotic yoghurts (Table 6.12).

Yoghurt containing unadapted *B. bifidum* and *B. breve* was estimated to have a shelf-life of 0 days, as the standard plate counts of both strains fell below the recommended minimum probiotic level in yoghurt (5.0 log CFU/g and 4.5 log CFU/g, respectively) (Table 6.12 and Figure 6.4). However, the adaptation of these species enhanced their culturability and, thus, their viability, extending the shelf-life to more than 0 days (Table 6.12). Initially, the adapted *B. bifidum* and *B. breve* strains demonstrated 6.0 and 6.8 log CFU/g viabilities, respectively (Figure 6.4). Despite this, their counts rapidly dropped below the recommended probiotic level by day 7, to 4.3 and 5.1 log CFU/g (Table 6.12 and Figure 6.4).

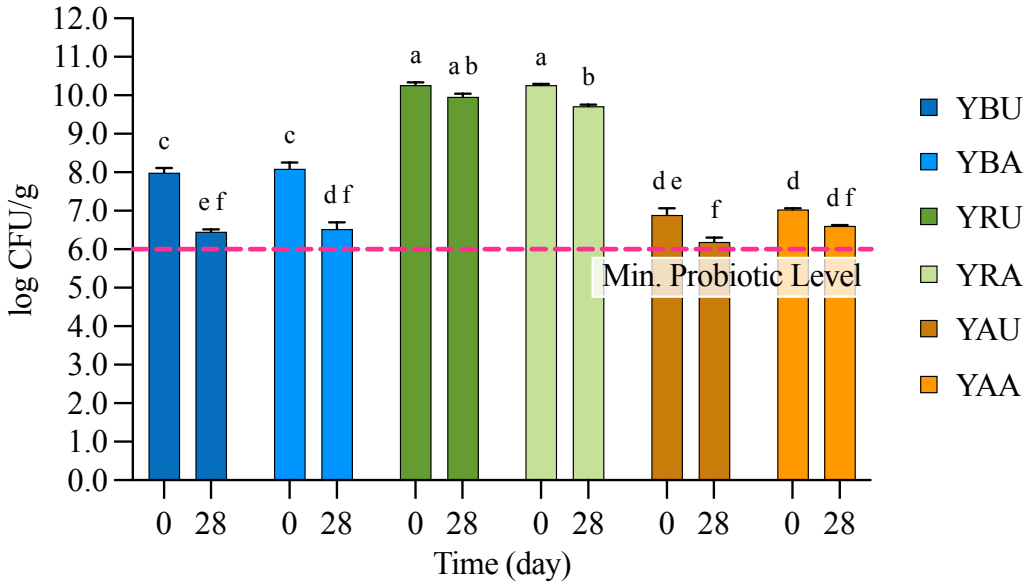


Figure 6.10 Probiotic viability in yoghurt prepared with unadapted or stress-adapted *B. bifidum, B. breve* or *B. animalis*, on day 0 and 28 of refrigerated storage (4 °C), as determined by PMAxx-qPCR. Values are the means ± standard deviation ($n = 2$). YBU: yoghurt with unadapted *B. bifidum*; YBA: yoghurt with stress-adapted *B. bifidum*; YRU: yoghurt with unadapted *B. breve*; YRA: yoghurt with stress-adapted *B. breve*; YAU: yoghurt with unadapted *B. animalis*; YAA: yoghurt with stress-adapted *B. animalis*.

Table 6.11 The effect of stress adaptation on the viability proportion index (VPI) of *Bifidobacterium* spp. in yoghurt at the end of storage (day 28), as determined by PMAxx-qPCR.

Species	***B. bifidum***	***B. breve***	***B. animalis***
Unadapted	0.808 ± 0.004	0.971 ± 0.015	0.900 ± 0.037
Stress-adapted	0.806 ± 0.006	0.947 ± 0.002	0.940 ± 0.007

Values are the means ± standard deviation ($n = 4$).

Remarkably, the shelf-life of yoghurts with *B. animalis* was estimated as 28 days, maintaining consistently high probiotic viability throughout the entire shelf-life period, irrespective of adaptation (Table 6.12). Unexpectedly, the PMAxx-qPCR counts of *Bifidobacterium* spp. remained above the recommended minimum level for probiotics in yoghurt at both the beginning (day 0) and end (day 28) of storage, resulting in a shelf-life of 28 days for all the yoghurt samples, irrespective of *Bifidobacterium* species or adaptation treatment (Table 6.12).

Table 6.12 Effect of stress adaptation on the predicted shelf-life of probiotic yoghurt containing *B. bifidum, B. breve* or *B. animalis*, based on the recommended minimum viable level for probiotics in yoghurt.

***Bifidobacterium* spp. in yoghurt**	**Shelf-life based on MRS-NNLP (days)**		**Shelf-life based on PMAxx-qPCR (days)**	
	Unadapted	**Stress Adapted**	**Unadapted**	**Stress Adapted**
B. bifidum	0	< 7	28	28
B. breve	0	< 7	28	28
B. animalis	28	28	28	28

[1]Shelf-life was established based on the minimum viable count requirement of 6 log CFU/g, as mandated by CODEX STAN 243-2003 (FAO/WHO, 2002). This criterion applies to micro-organisms that are added as supplementary cultures to yoghurt dairy products, beyond the primary starter culture, whereby a content claim can be made in the labelling.

6.4 DISCUSSION

In previous chapters (Chapters 4 and 5), the focus was solely on the oxidative stress challenges faced by the *Bifidobacterium* spp. included in this study. The present chapter expands this scope, exposing both unadapted and adapted *B. bifidum*, *B. breve* and *B. animalis* to the broad spectrum of environmental stressors encountered during yoghurt fermentation and storage. These include heat-, acid-, and cold stress (Chapter 2) (Sibanda et al., 2024). The viability assessments of these bacteria utilised two different enumeration methods: SPC and PMAxx-qPCR. This study also explored how the unadapted and adapted *Bifidobacterium* spp. influenced the physicochemical characteristics of yoghurt and the viability of its starter cultures. To our knowledge, this was the first investigation into the viability of oxidative stress-adapted *Bifidobacterium* spp. throughout the shelf-life of yoghurt, which made interpreting the results especially complex. The primary objective of this study and the thesis was to evaluate the hypothesis that the viability of *Bifidobacterium* spp. can be enhanced through adaptation to oxidative stress, thereby maintaining therapeutically appropriate levels of this probiotic in yoghurt during its fermentation and throughout its 28-day refrigerated shelf-life.

6.4.1 Yoghurt fermentation kinetics affected by stress-adapted *Bifidobacterium* species

The rapid decline in pH during fermentation likely resulted in the loss of viability of unadapted *B. bifidum* and *B. breve*. It is possible that the acidification of the yoghurt medium was more rapid than the ability of these cultures to adapt to the environment, effectively resulting in a loss in viability by the end of fermentation. These findings are in alignment with the observations made by El-Dieb et al. (2012), who reported a decline in *B. bifidum* levels during yoghurt fermentation, attributed to the high acidification rates of the yoghurt during fermentation. Although the acid tolerance and stress response mechanisms of *Bifidobacterium* spp. have been thoroughly investigated in previous research (Wei et al., 2019; Schöpping et al., 2022), the ability to maintain their viability during yoghurt production continues to present challenges (see Chapter 2). These findings underscore the inherent acid sensitivity of these species, a significant stress factor impacting their survival throughout yoghurt fermentation.

In turn, the gradual decline in pH in yoghurts containing adapted *Bifidobacterium* spp. created a more stable fermentation environment, enhancing bacterial survival and growth (Maus and Ingham, 2003). The adaptation treatment, potentially conferring cross-protection against multiple stress factors, allowed for more regulated nutrient utilisation and a controlled metabolic response of the probiotics throughout fermentation (Maus and Ingham, 2003; Jin et al., 2015). As a result, adapted *B. bifidum* retained high plate counts, while adapted *B. breve* showed an increase, indicating that the adaptation treatment provided these species with a metabolic and physiological advantage during the fermentation of yoghurt.

Interestingly, both unadapted and adapted *B. animalis* showed an increase in viability during fermentation, suggesting that this species possesses intrinsic mechanisms that confer resilience in the fermentative environment, regardless of adaptation. This could indicate a naturally higher tolerance to acidic conditions or an ability to rapidly adapt to changing environments, which could be an advantageous trait for probiotic formulations.

6.4.2 Shelf-life of probiotic yoghurt: Physicochemical and bacteriological properties of yoghurt as affected by stress-adapted *Bifidobacterium* species

The observed increase in TA in yoghurts containing unadapted *Bifidobacterium* spp. throughout storage, compared to those with adapted *Bifidobacterium* spp., contrasts with the findings of Oguz et al. (2023). They reported higher acidification in yoghurts containing acid-adapted strains of *Bifidobacterium* species. It is possible that the adaptation treatment of the current study led to the species developing mechanisms that manage acid production more efficiently to avoid excessive acidification, which could detrimentally affect their survival in the long term (Schöpping et al., 2022). Conversely, the unadapted strains, facing sudden exposure to the acidic yoghurt environment, might overcompensate by increasing their metabolic activity and organic acid production as a stress response, as discussed previously. Further research is needed to investigate any cross-protection resulting from the adaptation treatment against other stress factors that affect *Bifidobacterium* spp. viability, especially against acid, would confirm these speculations.

Furthermore, this study's findings suggest that the adaptation of *Bifidobacterium* spp. result in a more stable and potentially favourable yoghurt environment during storage. Adapted *Bifidobacterium* spp. seem to manage acid production in the yoghurts that avoid excessive acidity, which could be beneficial for maintaining the quality and sensory attributes of the yoghurt over time (Settachaimongkon et al., 2015). These findings call for investigating the stability of quality characteristics associated with yoghurt products through sensory analysis. However, confirming the safety of the adapted *Bifidobacterium* spp. is still required. Additionally, the analysis of syneresis would potentially provide additional information on the textural stability of yoghurts prepared with adapted *Bifidobacterium* spp., as discussed in subsequent sections.

The ORP of a product, such as a yoghurt, is not a direct measurement of the dissolved oxygen content but rather provides an indication of the redox balance of the medium (Bulat and Topcu, 2019). However, a high ORP level in the yoghurt could indicate an environment that favours the generation of ROS, which in turn would exert oxidative stress on the *Bifidobacterium* spp. in the medium.

The general increase in ORP across yoghurt samples during storage can be attributed to the continuous metabolic activities of the yoghurt microbiota, coupled with the introduction of

oxygen into the medium over time (Martin-Dejardin et al., 2013). Numerous researchers have reported significant decreases in the antioxidant capacity of yoghurt during storage (Campos et al., 2017; Khan et al., 2020). A reduction in the antioxidant capacity of yoghurts could result in an increase in ORP up to the eventual depletion of antioxidants within the yoghurt system, which could be explained by the overall peak change in ORP by day 21 of storage, being a possible indication of this depletion of antioxidants. However, investigations into the antioxidant capacities of the yoghurt containing adapted *Bifidobacterium* spp. would prove beneficial in the elucidation of these findings.

In contrast to yoghurts containing *B. bifidum* and *B. breve*, the slower increase in ORP in yoghurts with *B. animalis*, especially the adapted, likely resulted from the slower metabolism of the probiotics and the species' inherent resistance to oxidative stress (Oberg et al., 2011; Schöpping et al., 2022). Furthermore, the adaptation treatment likely enhanced this intrinsic resistance by effectively inducing the stress response mechanisms associated with the detoxification of ROS (Sibanda et al., 2024). Consequently, this could have led to a permanent genetic change, enhancing the adapted *B. animalis*' tolerance to oxidative stress in the yoghurt. It may also have enhanced their ability to neutralise significant amounts of oxidative stress, acting as a ROS quencher. As a result, the adapted *B. animalis* maintained a more stable ORP in yoghurt during storage, more so than yoghurt containing the unadapted counterpart.

The minimal variation in syneresis among yoghurt samples containing different *Bifidobacterium* species, as well as between unadapted and adapted variants, and the consistent syneresis observed in all yoghurt samples during storage, could be attributed to the yoghurt stabiliser that was used uniformly across all samples. Additionally, the consistent counts of *S. thermophilus* in all yoghurt samples throughout storage could have contributed to the uniform syneresis observed in all samples. *S. thermophilus* is known to produce exopolysaccharides (EPSs), which contribute to the structural integrity of the yoghurt gel (Fanning et al., 2012; Laureys et al., 2016). Probiotic species that produce EPSs can increase the water retention capacity of yoghurt and contribute to the stability of the yoghurt gel structure. In this case, it appears that the three *Bifidobacterium* spp. and their adapted variants are not significant producers of EPSs, hence their limited effect on yoghurt syneresis.

The presence of *Bifidobacterium* spp. in the yoghurt, irrespective of adaptation treatment, did not affect the viability of *S. thermophilus* during storage. These findings are in accordance with several reports in the literature, highlighting the species' industrial stability during yoghurt storage (Settachaimongkon et al., 2015; Yerlikaya et al., 2021). Furthermore, the high bacterial level, compared to *L. bulgaricus*, results from a higher initial inoculum level of the species into the yoghurt before fermentation. This is a common practice nowadays, whereby manufacturers of commercial yoghurt starter cultures will deliberately provide higher levels of *S. thermophilus* in their products to reduce 'post-acidification' (Yerlikaya et al., 2021). Unfortunately, the specific bacterial counts for the starter culture used in this study were not disclosed by the manufacturers of the yoghurt starter culture.

Notably, the adaptation of *B. breve* resulted in potentially enhanced synergistic interactions with *L. bulgaricus*, as the latter species showed an increase in viability in the yoghurt with adapted *B. breve* during storage. It is possible that the adaptation treatment resulted in stress response mechanisms which directly benefitted *L. bulgaricus*. Similar results were noted by Yerlikaya et al. (2021), whereby the viability of *L. bulgaricus* improved in probiotic yoghurt products during storage. It is possible that the adaptation treatment of *B. breve* led to an up-regulation of BCAA biosynthesis, as discussed previously, a known stress response mechanism that could directly benefit *L. bulgaricus* (Ulmer et al., 2022). Increased levels of BCAAs in the yoghurt could have supported the growth and viability of *L. bulgaricus* by providing essential nutrients for bacterial replication.

The stable plate counts of both unadapted and adapted *B. animalis* throughout yoghurt storage reflect the species' intrinsic resistance to oxidative stress and additional stress factors within the yoghurt environment. These findings correlate to those of Lamoureux et al. (2002) who reported a stable *B. animalis* viability in yoghurt throughout storage, remaining well above 6.0 log CFU/g. Oberg et al. (2011, 2013, 2012, 2015) and Oberg and Broadbent (2016) extensively studied the potential and genetic basis of the inherent and inducible response of *Bifidobacterium* spp. to oxidative stress and reported the induction of the oxidative stress response of *Bifidobacterium* spp. to be inherently species- and even strain-specific.

Conversely, the stark decline in *B. bifidum* and *B. breve* levels within the first seven days of yoghurt storage was likely a result of 'post-acidification' and large changes in ORP observed during the same time period. Furthermore, metabolites produced by the yoghurt starter cultures,

such as lactic acid, H_2O_2, bacteriocins, and volatile compounds, might have also influenced the viability of these microorganisms (Mortazavian et al., 2011). Similarly, (Oguz et al., 2023) observed a decrease in probiotic viability with increased acidity. These findings underscore the sensitivity of *B. bifidum* and *B. breve* to post-fermentation acid stress, as well as any interactions with the yoghurt starter culture – highlighting the need for strategies to mitigate acidification and maintain probiotic viability in yoghurt during storage (Schöpping et al., 2022).

Hence, the higher plate counts of adapted *B. bifidum* and *B. breve* compared to the unadapted in the yoghurt for the duration of storage suggests that the adaptation treatment successfully enhanced the viability of these probiotics in the yoghurt. Numerous researchers have proven the potential of adaptation of *Bifidobacterium* spp. to a stress factor, notably oxidative stress, to result in variants able to tolerate greater doses of the stress factor (Mozzetti et al., 2010; Oberg and Broadbent, 2016). These findings indicate that the adaptation treatment not only improved the stress response but may also have provided cross-protection against additional stressors like acid stress, thereby improving the overall resilience of *B. bifidum* and *B. breve* (Maus and Ingham, 2003; Wei et al., 2019). The improved stress response of the adapted *B. bifidum* and *B. breve* likely resulted in a slight improvement in plate count viability retention.

On the other hand, the fact that there was only a slight improvement in plate count retention of these probiotics might indicate that the adaptation might only be transient and effective in enhancing tolerance during fermentation. Maus and Ingham (2003) suggested that the yoghurt fermentation process may reverse any enhancements in stress response mechanisms acquired during the stress adaptation treatment. However, the adaptive stress response of *Bifidobacterium* spp. depend greatly on the species and the particular stress factor (Maus and Ingham, 2003; Saarela et al., 2004; Mozzetti et al., 2010; Schöpping et al., 2022).

Alternatively, the observed decline in plate counts of *B. bifidum* and *B. breve* during storage may be attributed to a loss of culturability, potentially indicating a transition to a viable but non-culturable (VBNC) state (Lahtinen et al., 2008; Wendel, 2022). It is well-documented that probiotic bacteria added to fermented food products can lose culturability while still retaining characteristics typical of viable cells (Lahtinen et al., 2008). Furthermore, Amor et al. (2002) demonstrated that a subset of unculturable *Bifidobacterium* cells remained metabolically active. These observations underscore the necessity for further investigation into the ability of *Bifidobacterium* spp. to enter a VBNC state and maintain metabolic activity in this condition.

The PCA results effectively illustrate that the effect of the stress adaptation treatment on the physicochemical and bacteriological characteristics of the yoghurt is highly dependent on the specific *Bifidobacterium* species involved, underscoring the nuanced, species-specific response and adaptation strategies to oxidative and other stress factors encountered by the probiotics in the yoghurt (Settachaimongkon et al., 2015). With its intrinsic tolerance to both oxidative and acid stress, *B. animalis* was the only standout species to retain high viability in yoghurt even as the total acidity and ORP were increasing, regardless of its adaptation state.

The PCA results further confirm the robust tolerance of *B. animalis* to oxidative and other stress factors within yoghurt. Supporting this, Oberg et al. (2013) demonstrated the strong stress response and tolerance of *B. animalis* subsp. *lactis* DSM 10140, a close relative of the *B. animalis* strain used in this study, particularly to H_2O_2 stress. The absence of notable effects from the adaptation treatment also suggests that the treatment may not have been intense enough to induce permanent enhancements in oxidative stress tolerance. Exploring more rigorous or diverse stress adaptation strategies, such as higher stress doses or multi-stressor treatments (combining acid and oxidative stresses), could prove more effective. Additionally, genomic analysis of both unadapted and adapted *Bifidobacterium* spp. might confirm any lasting genetic adaptations in the adapted strains. These findings underscore the need for further research into tailored stress adaptation strategies for specific *Bifidobacterium* species.

6.4.3 PMAxx-qPCR improves the quantification of probiotic bacteria within yoghurt

Recently, there has been a significant surge in the development and implementation of culture-independent methods for bacterial quantification, paving the way for the development of novel, reliable, rapid, and accurate technologies and methodologies for this purpose (Sibanda et al., 2024). Among these advancements, the novel PMAxx-qPCR assay developed by Marole et al. (2024) stands out for its ability to accurately quantify bacteria within mixed-species yoghurt. As a result, the present study utilised the PMAxx-qPCR assay to achieve accurate bacterial quantification, highlighting its ability to enumerate unculturable yet viable bacterial cells (i.e. VBNC) within the yoghurt.

The PMAxx-qPCR assay indicated that all three *Bifidobacterium* spp. remained above the recommended minimum level for probiotics (6 log CFU/g (FAO/WHO, 2003)) in yoghurt throughout the entire duration of storage. This not only underscores the precision of the PMAxx-qPCR assay compared to traditional standard plate counts but also suggests a significant presence of cells in a VBNC state (Lahtinen et al., 2008; Dias et al., 2020; Marole et al., 2024). One can hypothetically speculate that the proportion of *Bifidobacterium* spp. cells that were in a VBNC state in the yoghurt at the start (day 0) and end (day 28) of storage could be reflected in the percentage relative mean difference between the standard plate counts and the PMAxx-qPCR counts during the Bland-Altman method of comparison (Gagnon et al., 2015). Based on these findings, we can, therefore, speculate that, on average, 27% of the *Bifidobacterium* spp. in the yoghurt was in a VBNC state. However, to confirm the presence of VBNC *Bifidobacterium* cells, further investigations would be necessary, utilising FC and fluorescence probes in addition to the current PMAxx-qPCR method (Guo et al., 2021).

Previous research has identified cellular structures that correlate to certain characteristics associated with VBNC cells in TEM micrographs of *Bifidobacterium* spp. under lethal oxidative stress (Chapter 5, section 5.4.4). Therefore, the sustained high viability of *B. breve*, regardless of adaptation, likely reflects its transition to a VBNC state as a survival strategy against oxidative and other stresses during storage (Lahtinen et al., 2006, 2008). These findings highlight the promising potential of using *B. breve* as a probiotic culture in the development of probiotic yoghurt. However, they also emphasize the need for further investigations to confirm the presence of VBNC cells.

However, the probiotic efficacy of *Bifidobacterium* cells in a VBNC state is yet to be thoroughly explored. According to the definition of probiotics, these organisms should retain their ability to deliver therapeutic benefits to the host upon consumption (FAO/WHO, 2002; Hill et al., 2014). Furthermore, the ability of the VBNC *Bifidobacterium* cells to regain their culturability remains to be studied. Consequently, this matter warrants further investigation to ensure the efficacy of probiotics added to yoghurt for any therapeutic benefits to the consumer.

The study also observed a decline in *B. bifidum* counts towards the end of storage, highlighting its sensitivity to oxidative stress and suggesting that stress adaptation did not confer enhanced tolerance or cross-protection (Schöpping et al., 2022). In contrast, *B. animalis* showed stability throughout yoghurt processing and storage, with minimal differences between

counts from standard plate counts and PMAxx-qPCR, indicating it did not lose culturability and likely did not enter a VBNC state as *B. bifidum* and *B. breve* did. Therefore, *B. animalis* shows promise for the development of yoghurt with added probiotics.

Initially, it was assumed that the viability of *Bifidobacterium* declined during yoghurt storage due to bacterial death. However, the findings from the PMAxx-qPCR assay challenge this assumption, suggesting that *Bifidobacterium* spp. do not diminish in number. Instead, *Bifidobacterium* cells predominantly lose their culturability while maintaining their viability. This discrepancy arises because most traditional viability assays rely on culture-based techniques, which do not detect VBNC cells.

To reiterate, the main objective of this study was to enhance the survival of *Bifidobacterium* spp. during yoghurt fermentation and storage using a stress adaptation treatment. This aimed to maintain *Bifidobacterium* spp. levels above the recommended level (6 log CFU/g viable cells) throughout the yoghurt shelf-life, in accordance with CODEX STAN 243-2003 (FAO/WHO, 2003). It is, therefore, essential for manufacturers to maintain the viability of these microorganisms at or above this threshold to validate health benefit claims on the product label. Based on the findings from the bacteriological analysis in this study, we determined the shelf-life of the yoghurts prepared with unadapted or adapted *Bifidobacterium* spp., ensuring their level within the yoghurt remained at or above the recommended minimum level throughout the designated shelf-life period.

Hence, the adaptation of *Bifidobacterium* spp. slightly improved their viability during yoghurt fermentation and storage, though the improvement was minimal. Specifically, yoghurt with unadapted *B. bifidum* and *B. breve* had a shelf-life of 0 days, while the yoghurt with the adapted counterparts lasted slightly longer, between 0 and 7 days. The adaptation treatment likely resulted in certain cellular adaptations, such as the upregulation of general stress response genes, the optimisation of metabolic pathways for more efficient use of carbohydrates, as well as the modification of the membrane composition (Oberg et al., 2013; Schöpping et al., 2022) (See Chapter 4 and 5). Interestingly, both unadapted and adapted *B. animalis* maintained viability above 6 log CFU/g throughout the entire 28-day shelf-life, indicating their suitability as probiotics in yoghurt.

However, the PMAxx-qPCR assay findings imply the transition of *Bifidobacterium* spp. to an unculturable state, contributing to the extended shelf-life as indicated by the PMAxx-qPCR counts. These results confirm the assay's accuracy in quantifying bacterial species in mixed-species yoghurt and emphasise the need for further research on the probiotic properties of *Bifidobacterium* spp. in the VBNC state. Reflecting on the core definition of probiotics, it is essential that they continue to offer health benefits to the host when consumed (FAO/WHO, 2002).

6.5 CONCLUSION

This study demonstrates that adapting *Bifidobacterium* to oxidative stress before inclusion in yoghurt boosts their survival and positively influences the fermentation process. Yoghurts containing adapted strains showed more controlled acidification, creating a stable environment and supporting probiotic survival and product quality. Adaptation also impacts the physicochemical properties of yoghurt, with adapted strains producing yoghurt with lower acidity levels over its storage period, which may improve sensory attributes and consumer acceptability. Furthermore, differences in ORP between yoghurts containing adapted and unadapted *B. animalis* cells suggest better oxidative stress management, helping to maintain the structural integrity and overall quality of yoghurt during storage.

The adapted *Bifidobacterium* spp. generally maintain higher viable counts throughout yoghurt's shelf-life compared to non-adapted strains. Notably, the study highlights species-specific responses, with *B. animalis* demonstrating superior adaptation and maintaining higher viability throughout the shelf-life of the yoghurt compared to *B. bifidum* and *B. breve*. Moreover, the chapter provides insight into the transition of *Bifidobacterium* spp. to a VBNC state, which explains the higher bacterial counts obtained during the PMAxx-qPCR method compared to standard plate counts. The high correlation between standard plate counts and PMAxx-qPCR results highlights the accuracy and reliability of the enumeration method used, as PMAxx-qPCR is capable of detecting both culturable and non-culturable cells, thereby providing a more comprehensive assessment of probiotic viability in yoghurt.

The research highlights key areas for further investigation, such as developing species-specific stress adaptation treatments for *Bifidobacterium* spp. and exploring multi-stress adaptations and genomic analyses to confirm permanent adaptations. It significantly advances knowledge of the impact of stress adaptation on *Bifidobacterium* spp. in yoghurt. This study paves the way for future research that could enhance the effectiveness and consumer appeal of probiotic yoghurts. The implications for the industry are significant, offering insights into manufacturing processes that can help maintain the therapeutic potential of probiotic yoghurt, ensuring consumer health benefits and aligning with regulatory standards for probiotic foods. This study sets the stage for further explorations that could lead to more robust, effective, and consumer-friendly probiotic yoghurts.

CHAPTER 7

General discussion

7.1 CRITICAL REVIEW OF METHODOLOGY

This study applied a two-step stress adaptation treatment on *Bifidobacterium* spp. to enhance their tolerance to oxidative stress. Initially, the cells were exposed to a sublethal dose of the stressor, a strategy designed to induce its adaptive stress response mechanisms without compromising bacterial viability. This sublethal treatment aimed to biochemically and physiologically prepare the cells for greater stress levels. Following this, the cells underwent a lethal stress exposure, where the sublethal H_2O_2-treated cells were subjected to a lethal dose of the stressor for three successive generations. The lethal treatment was intended to permanently manifest the adaptive responses induced during the sublethal treatment in the cells' physiological, morphological, and ultrastructural characteristics.

Although this shortened adaptation process proved effective within the constraints of this study, it deviates from the conventional method of gradually exposing cells to sublethal stress across many generations. For instance, Jiang et al. (2016) successfully isolated acid-resistant mutants of *B. longum* subsp. *longum* BBMN68 by subjecting them to acid stress through 50 repetitive subcultures. This extended exposure potentially allowed for more robust adaptation mechanisms to develop, enhancing the resilience and stability of the strains. The duration of the stress adaptation treatment in the present study may have limited the full potential of the stress adaptation mechanisms of *Bifidobacterium* species. However, this approach was justifiable given the circumstances and provided significant insights into the adaptive capabilities of *Bifidobacterium* spp. under oxidative stress. Future studies could explore the impacts of prolonged sublethal exposure to oxidative stress.

Due to practical and logistical restraints, H_2O_2 was chosen as the oxidative stress factor during the stress adaptation treatments over O_2. Despite O_2 being more relevant regarding oxygen inclusion during yoghurt fermentation and storage, H_2O_2 was used in the current study as it directly induced oxidative stress. This choice allowed for a more controlled and feasible appli-

cation of the stress adaptation treatment. Similarly, Oberg and Broadbent (2016) used varying concentrations of H_2O_2 as a representative oxidative stress factor. Using O_2 as the stress factor during stress adaptation, a concentration of 3 - 5 % is suggested as the sublethal treatment, based on the findings by Zuo et al. (2018). However, this approach requires specific laboratory equipment, such as an anaerobic chamber, which would allow for the controlled modification of the atmospheric gas composition during the experimental and incubation phases.

Flow cytometry with fluorescent probes was utilised to analyse the physiological response of the *Bifidobacterium* spp. to the H_2O_2 treatments and the subsequent lethal H_2O_2 challenge. A significant challenge arose from the staining protocol and handling of the cells during sample preparation, which inevitably exposed the *Bifidobacterium* cultures to oxygen. Despite efforts to minimise this, some exposure occurred, potentially affecting the results. Ideally, the handling and staining of cells should have been conducted in an anaerobic chamber to prevent oxygen exposure, which was unavailable. Consequently, oxygen exposure during the staining process might have caused some membrane damage and the formation of ROS, complicating the differentiation between live and dead cells. Furthermore, in the presence of oxygen, the CRG probe may have undergone auto-oxidation, which could have led to the unintended detection of ROS. Future use of this protocol should consider using an anaerobic workflow throughout the cell staining and handling processes to accurately reflect the intrinsic ROS levels and cell viability.

The FC analysis results suggested various changes in membrane compositions and intracellular oxidation states. Extending the investigation to include an analysis of membrane fatty acid composition in response to the stress adaptation treatment and the lethal challenge could have provided deeper insights into the lipid profiles of the *Bifidobacterium* cultures. Such an analysis would have helped confirm hypotheses related to membrane-associated adaptive responses to oxidative stress. Furthermore, incorporating genomic sequencing could identify mutations and gene expression changes contributing to enhanced oxidative stress tolerance. This approach involves sequencing wild-type and adapted strains to identify specific single nucleotide polymorphisms (SNPs) associated with physiological changes observed during FC. Complementing this, proteomic studies, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), would be invaluable in identifying increased synthesis of proteins involved in the stress response of *Bifidobacterium* species. These protein profiles would validate the genomic data and provide deeper insights into the physiological adaptations of the

stress response. Additionally, exploring the production and role of EPSs could shed light on their protective functions under oxidative conditions, as EPSs has been implicated in cellular responses to environmental stresses.

Moreover, conducting standard plate counting alongside the FC analysis would have offered insights into the culturability and viability of the cells, providing potential evidence of the likely transition into a VBNC state under stressful conditions. Using a metabolic activity indicator, such as 5 (and 6)-carboxyfluorescein diacetate (cFDA) counterstained with PI, could have shed light on how *Bifidobacterium* spp. adapt metabolically, alongside their physiological and morphological changes, to stress adaptation treatment and oxidative stress.

Any enhanced adaptive stress response mechanisms associated with cell physiology or morphology were assessed by exposing the unadapted and H_2O_2-treated *Bifidobacterium* spp. to a lethal challenge of 1 mM H_2O_2 before conducting FC and microscopic analysis. However, this H_2O_2 concentration may not have been sufficient to constitute a lethal dose of oxidative stress. For comparison, Oberg et al. (2013) used higher H_2O_2 concentrations, ranging from 2.55 to 5.25 mM, for lethal challenges in experiments with two *B. lactis* strains.

The growth rates of unadapted and stress-adapted *Bifidobacterium* spp. under varying levels of oxidative stress were calculated assuming linear bacterial growth within the first 12 hours of incubation, which was justified by the exponential growth phase of the cells upon inoculation and supported by linear increases in OD_{600nm}, when including measurements at 3 and 6 hours. Despite efforts to minimise oxygen exposure, using anaerobic jars and oxygen-scavenging sachets, the complete exclusion of oxygen at each measurement was challenging. Although minimised to less than one minute per measurement and managed by frequent replacement of sachets, this exposure could have affected the growth kinetics results.

A significant methodological enhancement could be achieved by using an anaerobic chamber equipped with technology to automate the OD_{600nm} measurements, such as the use of an Atmospheric Control Unit (ACU, BMG LABTECH, Ortenberg, Germany). This unit is specifically designed to create and maintain an anaerobic environment, thus ensuring precise regulation of atmospheric conditions throughout the experiment. Implementing such systems would permit continuous and non-disruptive measurement of OD_{600nm}, enhancing the accuracy of growth curves and eliminating the risk of oxygen exposure during sampling. Additionally, fac-

tors such as cell size, shape, and the presence of particulates might have influenced the OD_{600nm} readings. Coupling these measurements with standard plate counting could have provided a more robust validation of the growth kinetics observations.

The viability of the unadapted and stress-adapted *Bifidobacterium* spp. was determined using standard plate count methods and the more advanced PMAxx-qPCR methods. While plate counting remains the traditional method for bacterial quantification, PMAxx-qPCR offers greater accuracy and faster results. Additionally, this novel method allowed for the detection of VBNC cells. However, the high cost and limited availability of PMAxx dye constrained our analysis allowing viability assessments only on days 0 and 28 of yoghurt storage. Consequently, this limitation may have omitted crucial data from days 7 to 21. Utilising plate counts in this study offered a traditional culturability perspective crucial for food safety standards, while PMAxx-qPCR provided modern molecular insights into overall cellular viability, which included the VBNC states.

7.2 MAIN RESEARCH FINDINGS

To exert its therapeutic benefits, *Bifidobacterium* spp. must maintain viability above 6 log CFU/g in yoghurt – a challenge due to its sensitivity to oxidative stress often introduced during production and storage. The current study explored oxidative stress adaptation to improve the tolerance of *B. bifidum*, *B. breve*, and *B. animalis* to oxidative stress, aiming to enhance their viability during yoghurt production and storage. This study confirmed that combined sublethal and subsequent lethal H_2O_2 treatments significantly influenced the oxidative stress responses and subsequent survival of *B. bifidum*, *B. breve*, and *B. animalis* during yoghurt fermentation and shelf-life. The H_2O_2 treatments led to notable physiological, morphological, and intracellular ultrastructural adaptations in the *Bifidobacterium* species, which were further enhanced after exposure to the lethal H_2O_2 challenge.

These adaptations included changes in membrane integrity and oxidation states of the cells, as indicated during FC analysis. Namely, integrating fatty acids into the lipid membranes during stress adaptation treatments improved membrane integrity by reducing fluidity and permeability to O_2 and ROS. This enhancement further suggests that cellular repair mechanisms

were activated, further bolstering the cells' resilience against oxidative stress. Additionally, using CRG to assess intracellular oxidation states after exposure to the lethal H_2O_2 challenge indicated an improved management of the intracellular redox balance, likely due to the activation of ROS detoxification enzymes, TrxR and AhpC. Overall, *B. animalis* exhibited the greatest retention of membrane integrity and reduced oxidation states following H_2O_2 treatments and exposure to the H_2O_2 lethal challenge, reflecting its inherent greater tolerance to oxidative stress. However, it should be noted that the findings from FC only provide indirect evidence of the physiological states of *Bifidobacterium* cells, thus only partially supporting the aforementioned speculations. Further investigations are required to directly confirm these adaptations and responses to stress adaptation and oxidative stress.

The physiological characterisations, particularly through supplementing with electron microscopy, provided insights into the cellular mechanisms supporting these observations. Notably, the expression of EVs and significant changes in cell shape among the stress-adapted *Bifidobacterium* spp. were markedly intensified following exposure to lethal H_2O_2 challenges. This enhancement of cellular adaptations under extreme conditions underscores their potential role in the oxidative stress response. However, the exact mechanisms by which these adaptations contribute to oxidative stress resistance remain to be fully elucidated. Ultrastructural observations further indicated compaction of cellular components, which could be a strategy to minimise the volume of cellular components exposed to ROS, thereby reducing the overall burden of oxidative stress on the cell. This compaction may also facilitate more efficient cellular repair processes by positioning enzymes and substrates closer to sites of oxidative damage.

Observations of physiological, morphological, and ultrastructural adaptations during H_2O_2 treatment and subsequent lethal H_2O_2 challenge have highlighted the oxidative stress response mechanisms that enhanced *Bifidobacterium* spp. viability during yoghurt fermentation and shelf-life. The stress adaptation treatment employed did indeed result in enhanced viability retention of the adapted variants during refrigerated storage, as evidenced by the standard plate counts. While adapted *B. bifidum* and *B. breve* showed improved viability and metabolic benefits, *B. animalis* displayed inherent resilience against oxidative stress regardless of adaptation. This stability underscores the species' intrinsic resistance to oxidative and other environmental stress (Oberg and Broadbent, 2016).

Consequently, the enhanced viability of the stress-adapted *Bifidobacterium* spp. prolonged the shelf-life of the probiotic yoghurt based on the minimum recommended viable level for probiotics in yoghurt (6 log CFU/g (FAO/WHO, 2003)). Moreover, the adaptation treatment may provide cross-protection against other stress factors, likely facilitating more regulated nutrient utilisation and controlled metabolic responses during fermentation. This potentially enhanced the metabolic capability of adapted *Bifidobacterium* spp. could also benefit yoghurt's overall quality and sensory attributes, a potential area for further research.

The findings from the PMAxx-qPCR assay, which showed higher *Bifidobacterium* spp. viability compared to standard plate counts, suggests that the observed decline in bacterial counts may not be due to cellular death but rather a loss in the culturability of the *Bifidobacterium* cells. According to Marole et al. (2024), the PMAxx-qPCR method can detect and quantify VBNC cells, which remain metabolically active yet lose their culturability under stressful conditions, such as yoghurt fermentation and storage. The discrepancy between declining MRS-NNLP counts and stable PMAxx-qPCR counts during yoghurt storage indicated that *Bifidobacterium* spp. lost their culturability and potentially transitioned into a VBNC state in response to the stressful environmental conditions encountered in the yoghurt environment. However, additional investigations would be necessary to confirm this hypothesis. While the methods of VBNC cellular states are underdeveloped, FC coupled with fluorogenic probes could be utilised to explore further and validate this transition into the VBNC state.

CHAPTER 8

Conclusions and future recommendations

This thesis explored the impact of oxidative stress adaptation treatments on the physiological responses of *B. bifidum*, *B. breve*, and *B. animalis*, aiming to enhance their tolerance to oxidative stress and improve viability throughout yoghurt fermentation and storage. The physiological response to the H_2O_2 treatments varies considerably among the *Bifidobacterium* species, potentially reflecting the strain variability that influences the tolerance to oxidative stress. For the intrinsically aerotolerant *B. animalis,* a high degree of cellular integrity is maintained under lethal oxidative stress regardless of the adaptation treatment. For *B. bifidum* and *B. breve*, the treatment leads to better retention of membrane integrity and maintenance of intracellular redox state, suggesting that these species could possess inducible mechanisms to combat oxidative stress. This adaptation likely involves strengthening the cell membrane through enhanced lipid synthesis and incorporation into the cellular membrane, which is critical for resisting ROS. This variability in the response to oxidative stress adaptation necessitates the development of tailored adaptation treatments based on the unique genetic and physiological characteristics of each *Bifidobacterium* species and even strain. Notwithstanding the variability, the practical implications of these findings suggest that oxidative stress adaptation could be a feasible method to improve the survivability and functional stability of susceptible probiotic *Bifidobacterium* species in yoghurt. Notably, the application of the PMAxx-qPCR viability method shows that the stress-susceptible *Bifidobacterium* species develop the VBNC state in yoghurt that potentially results in an underestimation of viability by the plate count method. This approach not only supports the maintenance of the minimum recommended probiotic levels (6 log CFU/g viable cells) throughout production and storage but also potentially extends the probiotic shelf-life of the yoghurt.

To fully leverage the oxidative stress adaptation in commercial probiotic applications, future research is recommended to explore the nuances of stress adaptation in *Bifidobacterium* spp. and should focus on confirming and clarifying species- and strain-specific changes in membrane composition and internal cellular responses to oxidative stress. A transcriptomic study

comparing the transcription rates of genes involved in oxidative stress response to those in other bacterial examples could provide valuable insights into the specific pathways and processes employed by *Bifidobacterium* spp. under oxidative stress. This should include the development of tailored stress adaptation treatments that address species-specific oxidative stress responses. More detailed exploration is needed to investigate the loss in culturability and potential transition into a VBNC state in *Bifidobacterium* spp. under environments of stress. Determining whether these cells can still exert their therapeutic probiotic effects on the host is crucial. This will allow a better understanding of their survival strategies and functional capabilities under stress. Moreover, exploring cross-protection against other stress factors, the stability of sensory characteristics in yoghurt, and antioxidant capacities post-adaptation will be crucial. A critical area of investigation is the impact of stress adaptation on the probiotic qualities of these strains. Since adaptation treatments could yield variants with altered therapeutic effects, assessing whether these adapted strains retain their beneficial probiotic characteristics is essential. While oxidative stress adaptation presents a promising approach for enhancing the tolerance of *Bifidobacterium* spp. to oxidative stress and, subsequently, their viability during yoghurt processing and storage, its implementation must be customised and assessed for each species to ensure it enhances, rather than diminishes, their beneficial therapeutic effects. Further research is essential to develop tailored, effective stress adaptation treatments that enhance the survival and efficacy of *Bifidobacterium* spp. in yoghurt, ensuring their safety and therapeutic benefits.

REFERENCES

Aakko, J., Sánchez, B., Gueimonde, M. and Salminen, S. (2014), 'Assessment of stress tolerance acquisition in the heat-tolerant derivative strains of Bifidobacterium animalis subsp. lactis BB-12 and Lactobacillus rhamnosus GG', *J Appl Microbiol* **117**(1), 239–48. Type: Journal Article.

Ahn, J. B., Hwang, H. J. and Park, J. H. (2001), 'Physiological responses of oxygen-tolerant anaerobic Bifidobacterium longum under oxygen', *Journal of Microbiology and Biotechnology* **11**, 443–451. Type: Journal Article.

Alessandri, G., van Sinderen, D. and Ventura, M. (2021), 'The genus Bifidobacterium: from genomics to functionality of an important component of the mammalian gut microbiota', *Computational and Structural Biotechnology Journal* **19**, 1472–1487. Type: Journal Article. **URL:** *https://www.sciencedirect.com/science/article/pii/S2001037021000787*

Alp, G. and Aslim, B. (2010), 'Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of Bifidobacterium spp. isolated from infants feces and breast milk', *Anaerobe* **16**(2), 101–5. Type: Journal Article.

Álvarez-Mercado, A. I., Plaza-Díaz, J., de Almagro, M. C., Gil, A., Moreno-Muńoz, J. A. and Fontana, L. (2022), 'Bifidobacterium longum subsp. infantis CECT 7210 Reduces Inflammatory Cytokine Secretion in Caco-2 Cells Cultured in the Presence of Escherichia coli CECT 515', *International Journal of Molecular Sciences* **23**(18), 10813. Type: Journal Article. **URL:** *https://www.proquest.com/docview/2716558690/abstract/72A663F226F34323PQ/1*

Alvarez-Ordóñez, A., Fernández, A., López, M., Arenas, R. and Bernardo, A. (2008), 'Modifications in membrane fatty acid composition of Salmonella typhimurium in response to growth conditions and their effect on heat resistance', *Int J Food Microbiol* **123**(3), 212–9. Type: Journal Article.

Amor, K. B., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., Vos, W. M. D. and Abee, T. (2002), 'Multiparametric Flow Cytometry and Cell Sorting for the

Assessment of Viable, Injured, and Dead Bifidobacterium Cells during Bile Salt Stress', *Applied and Environmental Microbiology* **68**(11), 5209–5216. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/aem.68.11.5209-5216.2002*

An, H., Douillard, F. P., Wang, G., Zhai, Z., Yang, J., Song, S., Cui, J., Ren, F., Luo, Y., Zhang, B. and Hao, Y. (2014), 'Integrated transcriptomic and proteomic analysis of the bile stress response in a centenarian-originated probiotic Bifidobacterium longum BBMN68', *Mol Cell Proteomics* **13**(10), 2558–72. Type: Journal Article.

Arboleya, S., Watkins, C., Stanton, C. and Ross, R. P. (2016), 'Gut Bifidobacteria Populations in Human Health and Aging', *Frontiers in Microbiology* **7**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2016.01204*

Averina, O. V., Poluektova, E. U., Marsova, M. V. and Danilenko, V. N. (2021), 'Biomarkers and Utility of the Antioxidant Potential of Probiotic Lactobacilli and Bifidobacteria as Representatives of the Human Gut Microbiota', *Biomedicines* **9**(10). Type: Journal Article.

Bazinet, L., Péricou, J. and Araya-Farias, M. (2009), 'Effect of flow rate and acid molarity on redox potential modulation during electroreduction of milk and simulated milk aqueous mineral phase', *Food Chemistry* **114**(3), 919–926. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0308814608012569*

Beheshtipour, H., Mortazavian, A. M., Haratian, P. and Darani, K. K. (2012), 'Effects of Chlorella vulgaris and Arthrospira platensis addition on viability of probiotic bacteria in yogurt and its biochemical properties', *European Food Research and Technology* **235**(4), 719–728. Type: Journal Article.
URL: *https://doi.org/10.1007/s00217-012-1798-4*

Berger, B., Moine, D., Mansourian, R. and Arigoni, F. (2010), 'HspR Mutations Are Naturally Selected in Bifidobacterium longum When Successive Heat Shock Treatments Are Applied', *Journal of Bacteriology* **192**(1), 256–263. Type: Journal Article.
URL: *https://journals.asm.org/doi/full/10.1128/jb.01147-09*

Biavati, B. and Mattarelli, P. (2006), The Family Bifidobacteriaceae, *in* 'The Prokaryotes :

Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes', New York, NY : Springer New York : Springer, pp. 322–382. Type: Book Section.

Booyens, J., Labuschagne, M. C. and Thantsha, M. S. (2014), 'In Vitro Antibacterial Mechanism of Action of Crude Garlic (Allium sativum) Clove Extract on Selected Probiotic Bifidobacterium Species as Revealed by SEM, TEM, and SDS-PAGE Analysis', *Probiotics and Antimicrobial Proteins* **6**(2), 82–87. Type: Journal Article.
URL: *https://doi.org/10.1007/s12602-013-9145-z*

Bose, S., Aggarwal, S., Singh, D. V. and Acharya, N. (2020), 'Extracellular vesicles: An emerging platform in gram-positive bacteria', *Microb Cell* **7**(12), 312–322. Type: Journal Article.

Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosens, N. and Morisset, D. (2014), 'Guidelines for validation of qualitative real-time PCR methods', *Trends in Food Science & Technology* **37**(2), 115–126. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0924224414000661*

Bulat, T. and Topcu, A. (2019), 'The effect of oxidation-reduction potential on the characteristics of UF white cheese produced using single strains of Lactococcus lactis', *LWT* **109**, 296–304. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0023643819303299*

Campos, D. C. D. S., Neves, L. T. B. C., Flach, A., Costa, L. A. M. A. and Sousa, B. O. D. (2017), 'POST-ACIDIFICATION AND EVALUATION OF ANTHOCYANINS STABILITY AND ANTIOXIDAN ACTIVITY IN AÇAI FERMENTED MILK AND YOGURTS (*Euterpe oleracea* Mart.)', *Revista Brasileira de Fruticultura* **39**. Type: Journal Article.

Candeias, L. P. and Steenken, S. (1993), 'Electron transfer in di(deoxy)nucleoside phosphates in aqueous solution: rapid migration of oxidative damage (via adenine) to guanine', *Journal of the American Chemical Society* **115**(6), 2437–2440. Type: Journal Article.
URL: *https://doi.org/10.1021/ja00059a044*

Chatterjee, N. and Walker, G. C. (2017), 'Mechanisms of DNA damage, repair, and mutagenesis', *Environ Mol Mutagen* **58**(5), 235–263. Type: Journal Article.

Citta, A., Folda, A., Scalcon, V., Scutari, G., Bindoli, A., Bellamio, M., Feller, E. and Rigobello, M. P. (2017), 'Oxidative changes in lipids, proteins, and antioxidants in yogurt during the shelf life', *Food Sci Nutr* **5**(6), 1079–1087. Type: Journal Article.

Corcoran, M. B., Stanton, C., Fitzgerald, G. and Ross, P. R. (2008), 'Life Under Stress: The Probiotic Stress Response and How it may be Manipulated', *Current Pharmaceutical Design* **14**(14), 1382–1399. Type: Journal Article.
URL: *http://www.eurekaselect.com/article/11948*

Cui, S. M., Zhao, J. X., Liu, X. M., Chen, Y. Q., Zhang, H. and Chen, W. (2016), 'Maximum-biomass concentration prediction for Bifidobacteria in the pH-controlled fed-batch culture', *Lett Appl Microbiol* **62**(3), 256–63. Type: Journal Article.

Dave, R. I. and Shah, N. P. (1997*a*), 'Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures', *International Dairy Journal* **7**(6), 435–443. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0958694697000265*

Dave, R. I. and Shah, N. P. (1997*b*), 'Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures', *International Dairy Journal* **7**(1), 31–41. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0958694696000465*

De Angelis, M. and Gobbetti, M. (2004), 'Environmental stress responses in Lactobacillus: a review', *Proteomics* **4**(1), 106–22. Type: Journal Article.

De Dea Lindner, J., Canchaya, C., Zhang, Z., Neviani, E., Fitzgerald, G. F., van Sinderen, D. and Ventura, M. (2008), 'Exploiting Bifidobacterium genomes: The molecular basis of stress response', *International journal of food microbiology.* **120**(1), 13. Type: Journal Article.

Derrien, M., Turroni, F., Ventura, M. and Sinderen, D. v. (2022), 'Insights into endogenous Bifidobacterium species in the human gut microbiota during adulthood', *Trends in Microbiology* **30**(10), 940–947. Type: Journal Article.
URL: *https://www.cell.com/trends/microbiology/abstract/S0966-842X(22)00094-4http://www.ncbi.nlm.nih.gov/pubmed/35577716*

Deshwal, G. K., Tiwari, S., Kumar, A., Raman, R. K. and Kadyan, S. (2021), 'Review on factors affecting and control of post-acidification in yoghurt and related products', *Trends in Food Science & Technology* **109**, 499–512. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0924224421000637*

Dhanashree, Rajashekharan, S., Krishnaswamy, B. and Kammara, R. (2017), 'Bifid Shape Is Intrinsic to Bifidobacterium adolescentis', *Front Microbiol* **8**, 478. Type: Journal Article.

Dias, C. O., Scariot, M. C., de Mello Castanho Amboni, R. D. and Arisi, A. C. M. (2020), 'Application of propidium monoazide coupled with quantitative PCR to evaluate cell viability of Bifidobacterium animalis subsp. lactis in a non-dairy probiotic beverage', *Annals of Microbiology* **70**(1), 22. Type: Journal Article.
URL: *https://doi.org/10.1186/s13213-020-01566-9*

Din, A. U., Hassan, A., Zhu, Y., Zhang, K., Wang, Y., Li, T., Wang, Y. and Wang, G. (2020), 'Inhibitory effect of Bifidobacterium bifidum ATCC 29521 on colitis and its mechanism', *The Journal of Nutritional Biochemistry* **79**, 108353. Type: Journal Article.

Dong, P., Yang, Y. and Wang, W. P. (2010), 'The role of intestinal bifidobacteria on immune system development in young rats', *Early Hum Dev* **86**(1), 51–8. Type: Journal Article.

du Toit, E., Vesterlund, S., Gueimonde, M. and Salminen, S. (2013), 'Assessment of the effect of stress-tolerance acquisition on some basic characteristics of specific probiotics', *Int J Food Microbiol* **165**(1), 51–6. Type: Journal Article.

Duranti, S., Ruiz, L., Lugli, G. A., Tames, H., Milani, C., Mancabelli, L., Mancino, W., Longhi, G., Carnevali, L., Sgoifo, A., Margolles, A., Ventura, M., Ruas-Madiedo, P. and Turroni, F. (2020), 'Bifidobacterium adolescentis as a key member of the human gut microbiota in the production of GABA', *Scientific Reports* **10**(1), 14112. Type: Journal Article.
URL: *https://doi.org/10.1038/s41598-020-70986-z*

Egan, M., Bottacini, F., O'Connell Motherway, M., Casey, P. G., Morrissey, R., Melgar, S., Faurie, J.-M., Chervaux, C., Smokvina, T. and van Sinderen, D. (2018), 'Staying alive: growth and survival of Bifidobacterium animalis subsp. animalis under in vitro and in vivo conditions', *Applied Microbiology and Biotechnology* **102**(24), 10645–10663. Type: Journal

Article.
URL: *https://doi.org/10.1007/s00253-018-9413-7*

El-Dieb, S. M., Abd Rabo, F. H. R., Badran, S. M., Abd El-Fattah, A. M. and Elshaghabee, F. M. F. (2012), 'The growth behaviour and enhancement of probiotic viability in bioyoghurt', *International Dairy Journal* **22**(1), 44–47. Type: Journal Article.
URL: *http://dx.doi.org/10.1016/j.idairyj.2011.08.003*

Evans, M. D., Dizdaroglu, M. and Cooke, M. S. (2004), 'Oxidative DNA damage and disease: induction, repair and significance', *Mutat Res* **567**(1), 1–61. Type: Journal Article.

Fallico, V., Rea, M., Stanton, C., Ilestam, N. and McKinney, J. (2020), 'Next-generation multiparameter flow cytometry assay improves the assessment of oxidative stress in probiotics', *Food Microbiology* **91**, 103501. Type: Journal Article.

Fan, X., Li, X., Zhang, T., Xu, J., Shi, Z., Wu, Z., Wu, J., Pan, D. and Du, L. (2021), 'A Novel qPCR Method for the Detection of Lactic Acid Bacteria in Fermented Milk', *Foods* **10**(12), 3066. Type: Journal Article.
URL: *https://www.mdpi.com/2304-8158/10/12/3066*

Fanning, S., Hall Lindsay, J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., O'Connell Motherway, M., Shanahan, F., Nally, K., Dougan, G. and van Sinderen, D. (2012), 'Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection', *Proceedings of the National Academy of Sciences* **109**(6), 2108–2113. Type: Journal Article.
URL: *https://doi.org/10.1073/pnas.1115621109*

FAO/WHO (2002), 'Guidelines for the evaluation of probiotics in food.Food and Health Agricultural Organization of the UnitedNations and World Health Organization.', *Working GroupReport* . Type: Journal Article.

FAO/WHO (2003), Standard for Fermented Milks (CXS 243-2003), Report, Codex Alimentarius Commission.

Fasnacht, M. and Polacek, N. (2021), 'Oxidative Stress in Bacteria and the Central Dogma of Molecular Biology', *Frontiers in Molecular Biosciences* **8**. Type: Journal Article.

URL: *https://www.frontiersin.org/articles/10.3389/fmolb.2021.671037*

Fenton, H. J. H. (1894), 'LXXIII.—Oxidation of tartaric acid in presence of iron', *Journal of the Chemical Society, Transactions* **65**(0), 899–910. Type: Journal Article.
URL: *http://dx.doi.org/10.1039/CT8946500899*

Fiocco, D., Longo, A., Arena, M. P., Russo, P., Spano, G. and Capozzi, V. (2020), 'How probiotics face food stress: They get by with a little help', *Crit Rev Food Sci Nutr* **60**(9), 1552–1580. Type: Journal Article.

Frakolaki, G., Giannou, V., Kekos, D. and Tzia, C. (2021), 'A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods', *Critical Reviews in Food Science and Nutrition* **61**(9), 1515–1536. Type: Journal Article.

Gagnon, M., Savard, P., Rivière, A., LaPointe, G. and Roy, D. (2015), 'Bioaccessible Antioxidants in Milk Fermented by *Bifidobacterium longum* subsp. *longum* Strains', *BioMed Research International* **2015**, 169381. Type: Journal Article.
URL: *https://doi.org/10.1155/2015/169381*

García-Cayuela, T., Tabasco, R., Peláez, C. and Requena, T. (2009), 'Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR', *International Dairy Journal* **19**(6), 405–409. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0958694609000296https://www.sciencedirect.com/science/article/pii/S0958694609000296?casa_token=245RuCu9qzsAAAAA:JCZZ0GNA0ej00wxtr_WJR5aRpQWY-NqmzlHpjgBW4FKg7092ttCydFK_soPBUSkhT_Ht5uQv4Q*

Gilbert, P., Collier, P. J. and Brown, M. R. (1990), 'Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response', *Antimicrob Agents Chemother* **34**(10), 1865–8. Type: Journal Article.

Grattepanche, F. and Lacroix, C. (2013), Production of viable probiotic cells, *in* B. McNeil, D. Archer, I. Giavasis and L. Harvey, eds, 'Microbial Production of Food Ingredients, Enzymes and Nutraceuticals', Woodhead Publishing, pp. 321–352. Type: Book Section.

URL: *https://www.sciencedirect.com/science/article/pii/B9780857093431500135*

Groeger, D., O'Mahony, L., Murphy, E. F., Bourke, J. F., Dinan, T. G., Kiely, B., Shanahan, F. and Quigley, E. M. M. (2013), 'Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut', *Gut Microbes* **4**(4), 325–339. Type: Journal Article.
URL: *https://doi.org/10.4161/gmic.25487*

Grogan, D. W. and Cronan, J. E. (1997), 'Cyclopropane ring formation in membrane lipids of bacteria', *Microbiology and Molecular Biology Reviews* **61**(4), 429–441. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/mmbr.61.4.429-441.1997*

Guo, L., Wan, K., Zhu, J., Ye, C., Chabi, K. and Yu, X. (2021), 'Detection and distribution of vbnc/viable pathogenic bacteria in full-scale drinking water treatment plants', *Journal of Hazardous Materials* **406**, 124335. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0304389420323256https://www.sciencedirect.com/science/article/pii/S0304389420323256?casa_token=c1HH3-ro_9UAAAAA:k-nOiiOLmfdrK3RrJTmcpUZip_X530s6cBqat26d9VTZ-YoyYFnmZpI_3PQnZcH6ADkvp4mNFg*

Hadjimbei, E., Botsaris, G. and Chrysostomou, S. (2022), 'Beneficial Effects of Yoghurts and Probiotic Fermented Milks and Their Functional Food Potential', *Foods* **11**(17), 2691. Type: Journal Article.
URL: *https://www.mdpi.com/2304-8158/11/17/2691*

Hamilton-Miller, J. M. (2004), 'Probiotics and prebiotics in the elderly', *Postgrad Med J* **80**(946), 447–51. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/15299153*

Hansen, C. (n.d.), 'Bifidobacterium, BB-12® - The world's most documented Bifidobacterium'. Type: Web Page.
URL: *https://www.chr-hansen.com/en/human-health-and-probiotics/our-probiotic-strains/bb-12*

He, B.-L., Xiong, Y., Hu, T.-G., Zong, M.-H. and Wu, H. (2023), 'Bifidobacterium spp. as functional foods: A review of current status, challenges, and strategies', *Critical Reviews in Food Science and Nutrition* **63**(26), 8048–8065. Type: Journal Article.

Hee, B. v. d. and Wells, J. M. (2021), 'Microbial Regulation of Host Physiology by Short-chain Fatty Acids', *Trends in Microbiology* **29**(8), 700–712. Type: Journal Article.
URL: *https://www.cell.com/trends/microbiology/abstract/S0966-842X(21)00035-4http://www.ncbi.nlm.nih.gov/pubmed/33674141, doi = 10.1016/j.tim.2021.02.001*

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C. and Sanders, M. E. (2014), 'The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic', *Nature Reviews Gastroenterology & Hepatology* **11**(8), 506–514. Type: Journal Article.
URL: *https://doi.org/10.1038/nrgastro.2014.66*

Hopkins, B. and Neumann, C. (2019), 'Redoxins as gatekeepers of the transcriptional oxidative stress response', *Redox Biology* **21**, 101104. Type: Journal Article.

Hsu, G. W., Ober, M., Carell, T. and Beese, L. S. (2004), 'Error-prone replication of oxidatively damaged DNA by a high-fidelity DNA polymerase', *Nature* **431**(7005), 217–221. Type: Journal Article.
URL: *https://doi.org/10.1038/nature02908*

Ibraheim, K., Ali, F. and Sorchee, S. (2020), 'Cellular and Molecular Biology Biosynthesis and characterization with antimicrobial activity of TiO2 nanoparticles using probiotic Bifidobacterium bifidum', *Cellular and molecular biology (Noisy-le-Grand, France)* **66**. Type: Journal Article.

IDF, I. O. f. S. I. and Federation, I. D. (2015), 'Milk and milk products - Starter cultures, probiotics and fermented products–Quantification of lactic acid bacteria by flow cytometry.', **ISO 19344: IDF 232**. Type: Journal Article.
URL: *https://www.iso.org/obp/ui/en/iso:std:iso:19344:ed-1:v1:en*

Ilha, E. C., Scariot, M. C., Treml, D., Pereira, T. P., Sant Anna, E. S., Prudêncio, E. S. and Arisi, A. C. M. (2016), 'Comparison of real-time PCR assay and plate count for Lactobacillus

paracasei enumeration in yoghurt', *Annals of Microbiology* **66**(2), 597–606. Type: Journal Article.
URL: *https://doi.org/10.1007/s13213-015-1137-7*

Imlay, J. A. (2008), 'Cellular Defenses against Superoxide and Hydrogen Peroxide', *Annual Review of Biochemistry* **77**(1), 755–776. Type: Journal Article.
URL: *https://doi.org/10.1146/annurev.biochem.77.061606.161055*

Inturri, R., Trovato, L., Volti, G. L., Oliveri, S. and Blandino, G. (2019), 'In vitro inhibitory activity of Bifidobacterium longum BB536 and Lactobacillus rhamnosus HN001 alone or in combination against bacterial and Candida reference strains and clinical isolates', *Heliyon* **5**(11), e02891. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S2405844019365508*

Jiang, Y., Ren, F., Liu, S., Zhao, L., Guo, H. and Hou, C. (2016), 'Enhanced Acid Tolerance in Bifidobacterium longum by Adaptive Evolution: Comparison of the Genes between the Acid-Resistant Variant and Wild-Type Strain', *Journal of Microbiology and Biotechnology* **26**(3), 452–460. Type: Journal Article.
URL: *http://www.jmb.or.kr/journal/view.html?doi=10.4014/jmb.1508.08030*

Jin, J., Qin, Q., Guo, H., Liu, S., Ge, S., Zhang, H., Cui, J. and Ren, F. (2015), 'Effect of Pre-Stressing on the Acid-Stress Response in Bifidobacterium Revealed Using Proteomic and Physiological Approaches', *PLOS ONE* **10**(2), e0117702. Type: Journal Article.
URL: *https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0117702*

Jin, J., Zhang, B., Guo, H., Cui, J., Jiang, L., Song, S., Sun, M. and Ren, F. (2012), 'Mechanism analysis of acid tolerance response of bifidobacterium longum subsp. longum BBMN 68 by gene expression profile using RNA-sequencing', *PLoS One* **7**(12), e50777. Type: Journal Article.

Juan, C. A., Pérez de la Lastra, J. M., Plou, F. J. and Pérez-Lebeña, E. (2021), 'The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies', *International Journal of*

Molecular Sciences **22**(9).
URL: *https://www.mdpi.com/1422-0067/22/9/4642*

Kawasaki, S., Satoh, T., Todoroki, M. and Niimura, Y. (2009), 'b-type dihydroorotate dehydrogenase is purified as a H2O2-forming NADH oxidase from Bifidobacterium bifidum', *Appl Environ Microbiol* **75**(3), 629–36. Type: Journal Article.

Kawasaki, S., Watanabe, M., Fukiya, S. and Yokota, A. (2018*a*), Chapter 7 - Stress Responses of Bifidobacteria: Oxygen and Bile Acid as the Stressors, *in* P. Mattarelli, B. Biavati, W. H. Holzapfel and B. J. B. Wood, eds, 'The Bifidobacteria and Related Organisms', Academic Press, pp. 131–143. Type: Book Section.
URL: *https://www.sciencedirect.com/science/article/pii/B9780128050606000077https://www.sciencedirect.com/science/article/abs/pii/B978012805060600077*

Kawasaki, S., Watanabe, M., Fukiya, S. and Yokota, A. (2018*b*), Stress Responses of Bifidobacteria: Oxygen and Bile Acid as the Stressors, *in* P. Mattarelli, B. Biavati, W. H. Holzapfel and B. J. B. Wood, eds, 'The Bifidobacteria and Related Organisms', Academic Press, pp. 131–143. Section: 7 Type: Book Section.
URL: *https://www.sciencedirect.com/science/article/pii/B978012805060600077*

Kelly, S. M., Munoz-Munoz, J. and van Sinderen, D. (2021), 'Plant Glycan Metabolism by Bifidobacteria', *Frontiers in Microbiology* **12**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2021.609418*

Khan, I. T., Nadeem, M., Imran, M. and Khalique, A. (2020), 'Impact of post fermentation cooling patterns on fatty acid profile, lipid oxidation and antioxidant features of cow and buffalo milk set yoghurt', *Lipids in Health and Disease* **19**(1), 74. Type: Journal Article.
URL: *https://doi.org/10.1186/s12944-020-01263-1*

Kim, J.-S., Chowdhury, N., Yamasaki, R. and Wood, T. K. (2018), 'Viable but non-culturable and persistence describe the same bacterial stress state', *Environmental Microbiology* **20**(6), 2038–2048. Type: Journal Article.
URL: *https://doi.org/10.1111/1462-2920.14075*

Klijn, A., Mercenier, A. and Arigoni, F. (2005), 'Lessons from the genomes of bifidobacteria', *FEMS Microbiology Reviews* **29**(3), 491–509. Type: Journal Article.
URL: *https://doi.org/10.1016/j.fmrre.2005.04.010*

Konieczna, I., Zarnowiec, P., Kwinkowski, M., Kolesinska, B., Fraczyk, J., Kaminski, Z. and Kaca, W. (2012), 'Bacterial urease and its role in long-lasting human diseases', *Curr Protein Pept Sci* **13**(8), 789–806. Type: Journal Article.

Kornberg, A. (1995), 'Inorganic polyphosphate: toward making a forgotten polymer unforgettable', *J Bacteriol* **177**(3), 491–6. Type: Journal Article.

Lahtinen, S. j., Ahokoski, H., Reinikainen, J. p., Gueimonde, M., Nurmi, J., Ouwehand, A. c. and Salminen, S. j. (2008), 'Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria', *Letters in Applied Microbiology* **46**(6), 693–698. Type: Journal Article.
URL: *https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1472-765X.2008.02374.xhttps://ami-journals.onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.2008.02374.x?casa_token=CWdz7iAmFU8AAAAA:Xu82NwhgtfPWOA9DOQ6HJ7cYj8iz6fSYlEp3HJk7J-JkLx4VgkqvOCkkUZIrBkaQKfJJtguywOyOGHy6*

Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P. and Salminen, S. J. (2006), 'Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product', *Food Microbiology* **23**(6), 571–577. Type: Journal Article.
URL: *http://dx.doi.org/10.1016/j.fm.2005.09.001*

Lamoureux, L., Roy, D. and Gauthier, S. (2002), 'Production of Oligosaccharides in Yogurt Containing Bifidobacteria and Yogurt Cultures', *Journal of dairy science* **85**, 1058–69. Type: Journal Article.

Laureys, D., Cnockaert, M., De Vuyst, L. and Vandamme, P. (2016), 'Bifidobacterium aquikefiri sp. nov., isolated from water kefir', *International Journal of Systematic and Evolutionary Microbiology* **66**(3), 1281–1286. Type: Journal Article.
URL: *https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijsem.0.000877*

Li, L.-Q., Chen, X., Zhu, J., Zhang, S., Chen, S.-Q., Liu, X., Li, L. and Yan, J.-K. (2023), 'Advances and challenges in interaction between heteroglycans and Bifidobacterium:

Utilization strategies, intestinal health and future perspectives', *Trends in Food Science & Technology* **134**, 112–122. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0924224423000754https://www.sciencedirect.com/science/article/pii/S0924224423000754?casa_token=uH-EreLh16sAAAAA:T9u-K5NONPha4Y-gC8xLXfv-h7QzQdHfhRR_-xxXIAAnUrp3ALst8WUjE2L34dpOtrVBxbinSg*

Li, S., Huang, R., Shah, N. P., Tao, X., Xiong, Y. and Wei, H. (2014), 'Antioxidant and antibacterial activities of exopolysaccharides from Bifidobacterium bifidum WBIN03 and Lactobacillus plantarum R315', *J Dairy Sci* **97**(12), 7334–43. Type: Journal Article.

Loquasto, J. R., Barrangou, R., Dudley, E. G. and Roberts, R. F. (2011), 'Short communication: the complete genome sequence of Bifidobacterium animalis subspecies animalis ATCC 25527(T) and comparative analysis of growth in milk with B. animalis subspecies lactis DSM 10140(T)', *J Dairy Sci* **94**(12), 5864–70. Type: Journal Article.

Lourens-Hattingh, A. and Viljoen, B. C. (2001), 'Yogurt as probiotic carrier food', *International Dairy Journal* **11**(1), 1–17. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S095869460100036X*

Lu, J. and Holmgren, A. (2014), 'The thioredoxin antioxidant system', *Free Radical Biology and Medicine* **66**, 75–87. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0891584913003808*

Margolles, A., García, L., Sánchez, B., Gueimonde, M. and de los Reyes-Gavilán, C. G. (2003), 'Characterisation of a Bifidobacterium strain with acquired resistance to cholate–a preliminary study', *Int J Food Microbiol* **82**(2), 191–8. Type: Journal Article.

Marole, T. A., Sibanda, T. and Buys, E. M. (2024), 'Assessing probiotic viability in mixed species yogurt using a novel propidium monoazide (PMAxx)-quantitative PCR method', *Frontiers in Microbiology* **15**. Type: Journal Article.
URL: *https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2024.1325268*

Martin-Dejardin, F., Ebel, B., Rojas, C., Gervais, P., Cayot, N. and Cachon, R. (2013), 'Redox Potential: Monitoring and Role in Development of Aroma Compounds, Rheological Properties and Survival of Oxygen Sensitive Strains During the Manufacture of Fermented Dairy Products', pp. 73–94. Type: Journal Article.

Matouskova, P., Hoova, J., Rysavka, P. and Marova, I. (2021), 'Stress Effect of Food Matrices on Viability of Probiotic Cells during Model Digestion', *Microorganisms* **9**(8), 1625. Type: Journal Article.
URL: *https://www.proquest.com/scholarly-journals/stress-effect-food-matrices-on-viability/docview/2565435806/se-2?accountid=14717*

Matsumoto, M., Ohishi, H. and Benno, Y. (2004), 'H+-ATPase activity in Bifidobacterium with special reference to acid tolerance', *Int J Food Microbiol* **93**(1), 109–13. Type: Journal Article.

Maus, J. and Ingham, S. (2003), 'Employment of stressful conditions during culture production to enhance subsequent cold- and acid-tolerance of bifidobacteria', *Journal of Applied Microbiology* **95**(1), 146–154. Type: Journal Article.
URL: *https://doi.org/10.1046/j.1365-2672.2003.01954.x*

Meile, L., Ludwig, W., Rueger, U., Gut, C., Kaufmann, P., Dasen, G., Wenger, S. and Teuber, M. (1997), 'Bifidobacterium lactis sp. nov., a Moderately Oxygen Tolerant Species Isolated from Fermented Milk', *Systematic and Applied Microbiology* **20**(1), 57–64. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0723202097800483*

Meybodi, N. M., Mortazavian, A. M., Arab, M. and Nematollahi, A. (2020), 'Probiotic viability in yoghurt: A review of influential factors', *International Dairy Journal* **109**. Type: Journal Article.

Mills, S., Stanton, C., Fitzgerald, G. F. and Ross, R. P. (2011), 'Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again', *Microb Cell Fact* **10 Suppl 1**, S19. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/21995734*

Mohedano, M. T., Konzock, O. and Chen, Y. (2022), 'Strategies to increase tolerance and robustness of industrial microorganisms', *Synthetic and Systems Biotechnology* **7**(1), 533–540. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S2405805X21000983*

Mortazavian, A., Ghorbanipour, S., Mohammadifar, m. a. and Mohammadi, M. (2011), 'Biochemical Properties and Viable Probiotic Population of Yogurt at Different Bacterial Inoculation Rates and Incubation Temperatures', *The Philippine Agricultural Scientist* **94**, 155–160. Type: Journal Article.

Mozaheb, N. and Mingeot-Leclercq, M. P. (2020), 'Membrane Vesicle Production as a Bacterial Defense Against Stress', *Front Microbiol* **11**, 600221. Type: Journal Article.

Mozzetti, V., Grattepanche, F., Moine, D., Berger, B., Rezzonico, E., Meile, L., Arigoni, F. and Lacroix, C. (2010), 'New method for selection of hydrogen peroxide adapted bifidobacteria cells using continuous culture and immobilized cell technology', *Microbial Cell Factories* **9**(1), 60. Type: Journal Article.
URL: *https://doi.org/10.1186/1475-2859-9-60*

Mukhopadhyay, A., Redding, A. M., Joachimiak, M. P., Arkin, A. P., Borglin, S. E., Dehal, P. S., Chakraborty, R., Geller, J. T., Hazen, T. C., He, Q., Joyner, D. C., Martin, V. J. J., Wall, J. D., Yang, Z. K., Zhou, J. and Keasling, J. D. (2007), 'Cell-Wide Responses to Low-Oxygen Exposure in *Desulfovibrio vulgaris* Hildenborough', *Journal of Bacteriology* **189**(16), 5996–6010. Type: Journal Article.
URL: *https://journals.asm.org/doi/10.1128/JB.00368-07*

Mättö, J., Malinen, E., Suihko, M. L., Alander, M., Palva, A. and Saarela, M. (2004), 'Genetic heterogeneity and functional properties of intestinal bifidobacteria', *J Appl Microbiol* **97**(3), 459–70. Type: Journal Article.

Nair, S. and Finkel, S. E. (2004), 'Dps protects cells against multiple stresses during stationary phase', *Journal of bacteriology* **186**(13), 4192–4198. Type: Journal Article.
URL: *https://pubmed.ncbi.nlm.nih.gov/15205421https://www.ncbi.nlm.nih.gov/pmc/articles/PMC421617/*

Neffe-Skocińska, K., Rzepkowska, A., Szydłowska, A., Kołożyn-Krajewska, D., Holban, A. and Grumezescu, A. (2018), 'Alternative and replacement foods'. Type: Generic.

Nishiyama, K., Takaki, T., Sugiyama, M., Fukuda, I., Aiso, M., Mukai, T., Odamaki, T., Xiao, J.-z., Osawa, R. and Okada, N. (2020), 'Extracellular Vesicles Produced by Bifidobacterium longum Export Mucin-Binding Proteins', *Applied and Environmental Microbiology* **86**(19). Type: Journal Article.

Njenga, R., Boele, J., Öztürk, Y. and Koch, H. G. (2023), 'Coping with stress: How bacteria fine-tune protein synthesis and protein transport', *J Biol Chem* **299**(9), 105163. Type: Journal Article.

Noriega, L., Gueimonde, M., Sánchez, B., Margolles, A. and de los Reyes-Gavilán, C. G. (2004), 'Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low PH and cross-resistance to bile salts in Bifidobacterium', *Int J Food Microbiol* **94**(1), 79–86. Type: Journal Article.

Novitsky, J. A. and Morita, R. Y. (1976), 'Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio', *Appl Environ Microbiol* **32**(4), 617–22. Type: Journal Article.

Nyanzi, R., Jooste, P. J. and Buys, E. M. (2021), 'Invited review: Probiotic yogurt quality criteria, regulatory framework, clinical evidence, and analytical aspects', *J Dairy Sci* **104**(1), 1–19. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/33348476*

Oberg, T. S. and Broadbent, J. R. (2016), Hydrogen Peroxide Resistance in Bifidobacterium Animalis Subsp. Lactis and Bifidobacterium Longum, *in* 'Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria', pp. 638–646. Type: Book Section.
URL: *https://doi.org/10.1002/9781119004813.ch61*

Oberg, T. S., Steele, J. L., Ingham, S. C., Smeianov, V. V., Briczinski, E. P., Abdalla, A. and Broadbent, J. R. (2011), 'Intrinsic and inducible resistance to hydrogen peroxide in Bifidobacterium species', *J Ind Microbiol Biotechnol* **38**(12), 1947–53. Type: Journal Article.

Oberg, T. S., Ward, R. E., Steele, J. L. and Broadbent, J. R. (2012), 'Identification of Plasmalogens in the Cytoplasmic Membrane of Bifidobacterium animalis subsp. lactis', *Applied and*

Environmental Microbiology **78**(3), 880–884. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/aem.06968-11*

Oberg, T. S., Ward, R. E., Steele, J. L. and Broadbent, J. R. (2013), 'Genetic and Physiological Responses of Bifidobacterium animalis subsp. lactis to Hydrogen Peroxide Stress', *Journal of Bacteriology* **195**(16), 3743–3751. Type: Journal Article.
URL: *https://doi.org/10.1128/jb.00279-13*

Oberg, T. S., Ward, R. E., Steele, J. L. and Broadbent, J. R. (2015), 'Transcriptome analysis of Bifidobacterium longum strains that show a differential response to hydrogen peroxide stress', *Journal of Biotechnology* **212**, 58–64. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0168165615300316*

O'Callaghan, A. and van Sinderen, D. (2016), 'Bifidobacteria and Their Role as Members of the Human Gut Microbiota', *Frontiers in Microbiology* **7**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2016.00925*

Oguz, S., Andic, S., Badak, N. and Demir, T. (2023), 'Determination of Probiotic Viability in Yoghurts Produced with Acid Adapted Lactobacillus acidophilus ATCC 4356 and Bifidobacterium bifidum ATCC 11863 During Refrigerated Storage'. Issue: 12 Pages: 2426-2434 Type: Electronic Article Volume: 11.
URL: *http://dx.doi.org/10.24925/turjaf.v11i12.2426-2434.6521*

Oren, A. (2024), 'On validly published names, correct names, and changes in the nomenclature of phyla and genera of prokaryotes: a guide for the perplexed', *npj Biofilms and Microbiomes* **10**, 20.
URL: *https://doi.org/10.1038/s41522-024-00494-9*

Ou, X., Cao, J., Cheng, A., Peppelenbosch, M. and Pan, Q. (2019), 'Errors in translational decoding: tRNA wobbling or misincorporation?', *PLoS Genetics* **15**. Type: Journal Article.

Palframan, R., Gibson, G. and Rastall, R. (2003), 'Carbohydrate Preferences of Bifidobacterium Species Isolated from the Human Gut', *Current issues in intestinal microbiology* **4**, 71–5. Type: Journal Article.

Pokusaeva, K., Fitzgerald, G. F. and van Sinderen, D. (2011), 'Carbohydrate metabolism in Bifidobacteria', *Genes & nutrition* **6**(3), 285–306. Type: Journal Article.
URL: *https://pubmed.ncbi.nlm.nih.gov/21484167https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3145055/*

Poole, L. B., Higuchi, M., Shimada, M., Calzi, M. L. and Kamio, Y. (2000), 'Streptococcus mutans H2O2-forming NADH oxidase is an alkyl hydroperoxide reductase protein', *Free Radic Biol Med* **28**(1), 108–20. Type: Journal Article.

Price, C. E., Reid, S. J., Driessen, A. J. and Abratt, V. R. (2006), 'The Bifidobacterium longum NCIMB 702259T ctr gene codes for a novel cholate transporter', *Appl Environ Microbiol* **72**(1), 923–6. Type: Journal Article.

Qian, Y., Borowski, W. J. and Calhoon, W. D. (2011), 'Intracellular granule formation in response to oxidative stress in Bifidobacterium', *International Journal of Food Microbiology* **145**(1), 320–325. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0168160510006562*

Rahman, M. M., Kim, W.-S., Kumura, H. and Shimazaki, K.-i. (2008), 'Autoaggregation and surface hydrophobicity of bifidobacteria', *World Journal of Microbiology and Biotechnology* **24**(8), 1593–1598. Type: Journal Article.
URL: *https://doi.org/10.1007/s11274-007-9650-x*

Rezzonico, E., Lariani, S., Barretto, C., Cuanoud, G., Giliberti, G., Delley, M., Arigoni, F. and Pessi, G. (2007), 'Global transcriptome analysis of the heat shock response of Bifidobacterium longum', *FEMS Microbiology Letters* **271**(1), 136–145. Type: Journal Article.
URL: *https://doi.org/10.1111/j.1574-6968.2007.00704.x*

Rosenberg, M., Azevedo, N. F. and Ivask, A. (2019), 'Propidium iodide staining underestimates viability of adherent bacterial cells', *Scientific Reports* **9**(1), 6483. Type: Journal Article.
URL: *https://doi.org/10.1038/s41598-019-42906-3*

Ruiz, L., Gueimonde, M., Ruas-Madiedo, P., Ribbera, A., Reyes-Gavilán, C. G. d. l., Ventura, M., Margolles, A. and Sánchez, B. (2012), 'Molecular Clues To Understand the Aerotolerance Phenotype of Bifidobacterium animalis subsp. lactis', *Applied and Environmental*

Microbiology **78**(3), 644–650. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/AEM.05455-11*

Ruiz, L., Sánchez, B., Ruas-Madiedo, P., de Los Reyes-Gavilán, C. G. and Margolles, A. (2007), 'Cell envelope changes in Bifidobacterium animalis ssp. lactis as a response to bile', *FEMS Microbiol Lett* **274**(2), 316–22. Type: Journal Article.

Saarela, M., Rantala, M., Hallamaa, K., Nohynek, L., Virkajärvi, I. and Mättö, J. (2004), 'Stationary-phase acid and heat treatments for improvement of the viability of probiotic lactobacilli and bifidobacteria', *J Appl Microbiol* **96**(6), 1205–14. Type: Journal Article.

Sánchez, B., Champomier-Vergès, M.-C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., Reyes-Gavilán, C. G. d. l., Johansen, E., Zagorec, M. and Margolles, A. (2007), 'Low-pH Adaptation and the Acid Tolerance Response of Bifidobacterium longum Biotype longum', *Applied and Environmental Microbiology* **73**(20), 6450–6459. Type: Journal Article.
URL: *https://doi.org/10.1128/AEM.00886-07*

Sarkar, S. (2019), 'Potentiality of probiotic yoghurt as a functional food – a review', *Nutrition & Food Science* **49**(2), 182–202. Type: Journal Article.
URL: *http://dx.doi.org/10.1108/NFS-05-2018-0139*

Satoh, T., Todoroki, M., Kobayashi, K., Niimura, Y. and Kawasaki, S. (2019), 'Purified thioredoxin reductase from O2-sensitive Bifidobacterium bifidum degrades H2O2 by interacting with alkyl hydroperoxide reductase', *Anaerobe* **57**, 45–54. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S1075996419300514*

Schiweck, C., Edwin Thanarajah, S., Aichholzer, M., Matura, S., Reif, A., Vrieze, E., Weigert, A. and Visekruna, A. (2022), 'Regulation of CD4+ and CD8+ T Cell Biology by Short-Chain Fatty Acids and Its Relevance for Autoimmune Pathology', *International Journal of Molecular Sciences* **23**(15), 8272. Type: Journal Article.
URL: *https://www.mdpi.com/1422-0067/23/15/8272*

Schmidt, G. and Zink, R. (2000), 'Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve', *Int J Food Microbiol* **55**(1-3), 41–5. Type: Journal Article.

Schöpping, M., Vesth, T., Jensen, K., Franzéén, C. J. and Zeidan, A. A. (2022), 'Genome-Wide Assessment of Stress-Associated Genes in Bifidobacteria', *Applied and Environmental Microbiology* **88**(7), e02251–21. Type: Journal Article.
URL: *https://journals.asm.org/doi/10.1128/aem.02251-21*

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 Syst Biol Appl* **7**(1), 47. Type: Journal Article.

Schöpping, M., Goel, A., Jensen, K., Faria, R. A., Franzén, C. J. and Zeidan, A. A. (2023), 'Novel Insights into the Molecular Mechanisms Underlying Robustness and Stability in Probiotic Bifidobacteria', *Applied and Environmental Microbiology* **89**(3), e00082–23. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/aem.00082-23*

Schöpping, M., Zeidan, A. A. and Franzén, C. J. (2022), 'Stress Response in Bifidobacteria', *Microbiology and Molecular Biology Reviews* **86**(4). Type: Journal Article.

Seixas, A. F., Quendera, A. P., Sousa, J. P., Silva, A. F. Q., Arraiano, C. M. and Andrade, J. M. (2022), 'Bacterial Response to Oxidative Stress and RNA Oxidation', *Frontiers in Genetics* **12**. Type: Journal Article.
URL: *https://www.frontiersin.org/journals/genetics/articles/10.3389/fgene.2021.821535*

Settachaimongkon, S., van Valenberg, H. J. F., Winata, V., Wang, X., Nout, M. J. R., van Hooijdonk, T. C. M., Zwietering, M. H. and Smid, E. J. (2015), 'Effect of sublethal preculturing on the survival of probiotics and metabolite formation in set-yoghurt', *Food Microbiology* **49**, 104–115. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0740002015000155*

Sfakianakis, P. and Tzia, C. (2014), 'Conventional and Innovative Processing of Milk for Yogurt Manufacture; Development of Texture and Flavor: A Review', *Foods* **3**(1), 176–193. Type: Journal Article.

Shah, N. P. (2000), 'Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods', *Journal of Dairy Science* **83**(4), 894–907. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0022030200749538*

Shah, N. P. (2011), BACTERIA, BENEFICIAL | Bifidobacterium spp.: Morphology and Physiology, *in* J. W. Fuquay, ed., 'Encyclopedia of Dairy Sciences (Second Edition)', Academic Press, San Diego, pp. 381–387. Type: Book Section.
URL: *https://www.sciencedirect.com/science/article/pii/B9780123744074000431*

Shang, J., Wan, F., Zhao, L., Meng, X. and Li, B. (2020), 'Potential Immunomodulatory Activity of a Selected Strain Bifidobacterium bifidum H3-R2 as Evidenced in vitro and in Immunosuppressed Mice', *Frontiers in Microbiology* **11**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2020.02089*

Shang, N., Xu, R. and Li, P. (2013), 'Structure characterization of an exopolysaccharide produced by Bifidobacterium animalis RH', *Carbohydr Polym* **91**(1), 128–34. Type: Journal Article.

Shcherbik, N. and Pestov, D. G. (2019), 'The Impact of Oxidative Stress on Ribosomes: From Injury to Regulation', *Cells* **8**(11). Type: Journal Article.

Shi, Z. (2019), 'Gut Microbiota: An Important Link between Western Diet and Chronic Diseases', *Nutrients* **11**(10). Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/31554269*

Sibanda, T., Marole, T. A., Thomashoff, U. L., Thantsha, M. S. and Buys, E. M. (2024), 'Bifidobacterium species viability in dairy-based probiotic foods: challenges and innovative approaches for accurate viability determination and monitoring of probiotic functionality', *Frontiers in Microbiology* **15**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2024.1327010*

Sies, H. and Jones, D. P. (2020), 'Reactive oxygen species (ROS) as pleiotropic physiological signalling agents', *Nature Reviews Molecular Cell Biology* **21**(7), 363–383. Type: Journal

Article.
URL: *https://doi.org/10.1038/s41580-020-0230-3*

Statista (2024), 'Yogurt - worldwide: Statista market forecast'. Type: Online Database.
URL: *https://www.statista.com/outlook/cmo/food/dairy-products-eggs/yogurt/worldwiderevenue*

Sugimoto, S., Abdullah Al, M. and Sonomoto, K. (2008), 'Molecular chaperones in lactic acid bacteria: physiological consequences and biochemical properties', *J Biosci Bioeng* **106**(4), 324–36. Type: Journal Article.

Sánchez, B., Champomier-Vergès, M.-C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., Reyes-Gavilán, C. G. d. l., Johansen, E., Zagorec, M. and Margolles, A. (2007), 'Adaptation and Response of *Bifidobacterium animalis* subsp. *lactis* to Bile: a Proteomic and Physiological Approach', *Applied and Environmental Microbiology* **73**(21), 6757–6767. _eprint: https://journals.asm.org/doi/pdf/10.1128/aem.00637-07.
URL: *https://journals.asm.org/doi/abs/10.1128/aem.00637-07*

Tacconi, S., Sgorbati, B., Modesto, M., Biavati, B., Nissen, L. and Mattarelli, P. (2012), 'Carbohydrate stress-related response in Bifidobacterium pseudolongum subsp. globosum', *Annals of Microbiology* **62**, 1751–1756. Type: Journal Article.

Talwalkar, A. and Kailasapathy, K. (2003), 'Metabolic and biochemical responses of probiotic bacteria to oxygen', *J Dairy Sci* **86**(8), 2537–46. Type: Journal Article.

Talwalkar, A. and Kailasapathy, K. (2004*a*), 'A Review of Oxygen Toxicity in Probiotic Yogurts: Influence on the Survival of Probiotic Bacteria and Protective Techniques', *Comprehensive reviews in food science and food safety* **3**(3), 117–124. Type: Journal Article.

Talwalkar, A. and Kailasapathy, K. (2004*b*), 'The role of oxygen in the viability of probiotic bacteria with reference to L. acidophilus and Bifidobacterium spp', *Curr Issues Intest Microbiol* **5**(1), 1–8. Type: Journal Article.

Talwalkar, A., Miller, C. W., Kailasapathy, K. and Nguyen, M. H. (2004), 'Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt', *International Journal of Food Science & Technology* **39**(6), 605–611. Type: Journal Article.
URL: *https://doi.org/10.1111/j.1365-2621.2004.00820.x*

Tanaka, K., Satoh, T., Kitahara, J., Uno, S., Nomura, I., Kano, Y., Suzuki, T., Niimura, Y. and Kawasaki, S. (2018), 'O2-inducible H2O2-forming NADPH oxidase is responsible for the hyper O2 sensitivity of Bifidobacterium longum subsp. infantis', *Scientific Reports* **8**(1), 10750. Type: Journal Article.
URL: *https://doi.org/10.1038/s41598-018-29030-4*

Teitelbaum, J. E. and Walker, W. A. (2002), 'Nutritional impact of pre- and probiotics as protective gastrointestinal organisms', *Annu Rev Nutr* **22**, 107–38. Type: Journal Article.

Thomashoff, U., Sibanda, T. and Buys, E. (2021), Probiotic Viability in a Synbiotic Yoghurt Incorporating Lactulose. Publisher: University of Pretoria.

Torrecilla, I., Ruggiano, A., Kiianitsa, K., Aljarbou, F., Lascaux, P., Hoslett, G., Song, W., Maizels, N. and Ramadan, K. (2024), 'Isolation and detection of DNA–protein crosslinks in mammalian cells', *Nucleic Acids Research* **52**(2), 525–547. Type: Journal Article.
URL: *https://doi.org/10.1093/nar/gkad1178*

Toure, R., Kheadr, E., Lacroix, C., Moroni, O. and Fliss, I. (2003), 'Production of antibacterial substances by bifidobacterial isolates from infant stool active against Listeria monocytogenes', *J Appl Microbiol* **95**(5), 1058–69. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/14633035*

Turroni, F., Taverniti, V., Ruas-Madiedo, P., Duranti, S., Guglielmetti, S., Lugli, G. A., Gioiosa, L., Palanza, P., Margolles, A., van Sinderen, D. and Ventura, M. (2014), 'Bifidobacterium bifidum PRL2010 Modulates the Host Innate Immune Response', *Applied and Environmental Microbiology* **80**(2), 730–740. Type: Journal Article.
URL: *https://journals.asm.org/doi/full/10.1128/aem.03313-13*

Ulmer, A., Erdemann, F., Mueller, S., Loesch, M., Wildt, S., Jensen, M., Gaspar, P., Zeidan, A. and Takors, R. (2022), 'Differential Amino Acid Uptake and Depletion in Mono-Cultures and Co-Cultures of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus in a Novel Semi-Synthetic Medium', *Microorganisms* **10**, 1771. Type: Journal Article.

Ultee, E., Ramijan, K., Dame, R. T., Briegel, A. and Claessen, D. (2019), Stress-induced adaptive morphogenesis in bacteria, *in* R. K. Poole, ed., 'Advances in Microbial Physiology', Vol. 74, Academic Press, pp. 97–141. Section: 2 Type: Book Section.

URL: *https://www.sciencedirect.com/science/article/pii/S0065291119300013*

Ventura, M., Canchaya, C., Zhang, Z., Bernini, V., Fitzgerald, G. F. and van Sinderen, D. (2006), 'How high G+C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators', *FEMS Microbiol Rev* **30**(5), 734–59. Type: Journal Article.

Ventura, M., Margolles, A., Turroni, F., Zomer, A., de los Reyes-Gavilán, C. G. and van Sinderen, D. (2011), Stress Responses of Bifidobacteria, *in* E. Tsakalidou and K. Papadimitriou, eds, 'Stress Responses of Lactic Acid Bacteria', Springer US, Boston, MA, pp. 323–347. Type: Book Section.
URL: *https://doi.org/10.1007/978-0-387-92771-8_14*

Ventura, M., Zink, R., Fitzgerald, G. F. and van Sinderen, D. (2005), 'Gene Structure and Transcriptional Organization of the dnaK Operon of Bifidobacterium breve UCC 2003 and Application of the Operon in Bifidobacterial Tracing', *Applied and Environmental Microbiology* **71**(1), 487–500. Type: Journal Article.
URL: *https://journals.asm.org/doi/full/10.1128/aem.71.1.487-500.2005*

Vernazza, C. L., Gibson, G. R. and Rastall, R. A. (2006), 'Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium', *Journal of Applied Microbiology* **100**(4), 846–853. Type: Journal Article.
URL: *https://ami-journals.onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2672.2006.02832.x*

Vlasova, A. N., Kandasamy, S., Chattha, K. S., Rajashekara, G. and Saif, L. J. (2016), 'Comparison of probiotic lactobacilli and bifidobacteria effects, immune responses and rotavirus vaccines and infection in different host species', *Veterinary Immunology and Immunopathology* **172**, 72–84. Epub 2016 Jan 14.

Waddington, L., Cyr, T., Hefford, M., Hansen, L. T. and Kalmokoff, M. (2010), 'Understanding the acid tolerance response of bifidobacteria', *J Appl Microbiol* **108**(4), 1408–20. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/19796122*

Wei, Y., Gao, J., Liu, D., Li, Y. and Liu, W. (2019), 'Adaptational changes in physiological and transcriptional responses of Bifidobacterium longum involved in acid stress resistance after successive batch cultures', *Microbial Cell Factories* **18**(1), 156. Type: Journal Article.
URL: *https://doi.org/10.1186/s12934-019-1206-x*

Wendel, U. (2022), 'Assessing Viability and Stress Tolerance of Probiotics—A Review', *Frontiers in Microbiology* **12**. Type: Journal Article.
URL: *https://www.frontiersin.org/articles/10.3389/fmicb.2021.818468*

Wesche, A. M., Gurtler, J. B., Marks, B. P. and Ryser, E. T. (2009), 'Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens', *J Food Prot* **72**(5), 1121–38. Type: Journal Article.

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A. and Jenkins, D. J. A. (2006), 'Colonic Health: Fermentation and Short Chain Fatty Acids', *Journal of Clinical Gastroenterology* **40**(3), 235–243. Type: Journal Article.

Woolworths (n.d.), 'HOW WE REINVENTED OUR YOGHURT'. Type: Web Page.
URL: *https://www.woolworths.co.za/content/article/woolies-buying-guides/how-we-reinvented-our-yoghurt/_/A-cmp207831*

Xiao, M., Xu, P., Zhao, J., Wang, Z., Zuo, F., Zhang, J., Ren, F., Li, P., Chen, S. and Ma, H. (2011), 'Oxidative stress-related responses of Bifidobacterium longum subsp. longum BBMN68 at the proteomic level after exposure to oxygen', *Microbiology (Reading)* **157**(Pt 6), 1573–1588. Type: Journal Article.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/21349974*

Yang, B., Ding, M., Chen, Y., Han, F., Yang, C., Zhao, J., Malard, P., Stanton, C., Ross, R. P., Zhang, H. and Chen, W. (2021), 'Development of gut microbiota and bifidobacterial communities of neonates in the first 6 weeks and their inheritance from mother', *Gut Microbes* **13**(1), 1–13. Type: Journal Article.

Yang, X., Hang, X., Tan, J. and Yang, H. (2015), 'Differences in acid tolerance between Bifidobacterium breve BB8 and its acid-resistant derivative B. breve BB8dpH, revealed by RNA-sequencing and physiological analysis', *Anaerobe* **33**, 76–84. Type: Journal Article.

Yerlikaya, O., Saygili, D. and Akpinar, A. (2021), 'An application of selected enterococci using Bifidobacterium animalis subsp. lactis BB-12 in set-style probiotic yoghurt-like products', *Food Bioscience* **41**. Type: Journal Article.
URL: *http://dx.doi.org/10.1016/j.fbio.2021.101096*

Yunes, R. A., Poluektova, E. U., Dyachkova, M. S., Klimina, K. M., Kovtun, A. S., Averina, O. V., Orlova, V. S. and Danilenko, V. N. (2016), 'GABA production and structure of gadB/gadC genes in Lactobacillus and Bifidobacterium strains from human microbiota', *Anaerobe* **42**, 197–204. Type: Journal Article.

Zeller, T. and Klug, G. (2006), 'Thioredoxins in bacteria: functions in oxidative stress response and regulation of thioredoxin genes', *Naturwissenschaften* **93**(6), 259–66. Type: Journal Article.

Zhang, H., Liu, M., Liu, X., Zhong, W., Li, Y., Ran, Y., Guo, L., Chen, X., Zhao, J., Wang, B. and Zhou, L. (2020), 'Bifidobacterium animalis ssp. Lactis 420 Mitigates Autoimmune Hepatitis Through Regulating Intestinal Barrier and Liver Immune Cells', *Frontiers in Immunology* **11**.
URL: *https://www.frontiersin.org/articles/10.3389/fimmu.2020.569104*

Zhang, J., Wang, S., Zeng, Z., Qin, Y. and Li, P. (2019), 'The complete genome sequence of Bifidobacterium animalis subsp. lactis 01 and its integral components of antioxidant defense system', *3 Biotech* **9**(10), 352. Type: Journal Article.
URL: *https://doi.org/10.1007/s13205-019-1890-6*

Zhang, J. et al. (2023*a*), 'Single-cell rapid identification, in situ viability and vitality profiling, and genome-based source-tracking for probiotics products', *iMeta* **2**(3), 117.
URL: *https://onlinelibrary.wiley.com/doi/abs/10.1002/imt2.117*

Zhang, T. et al. (2023*b*), 'From the past to the future: Fermented milks and their health effects against human diseases', *Food Frontiers* **4**(4), 1747–1777. Type: Journal Article.
URL: *https://doi.org/10.1002/fft2.304*

Zomer, A., Fernandez, M., Kearney, B., Fitzgerald, G. F., Ventura, M. and Sinderen, D. v. (2009), 'An Interactive Regulatory Network Controls Stress Response in *Bifidobacterium*

breve UCC2003', *Journal of Bacteriology* **191**(22), 7039–7049. Type: Journal Article.
URL: *https://journals.asm.org/doi/abs/10.1128/jb.00897-09*

Zuo, F., Yu, R., Khaskheli, G. B., Ma, H., Chen, L., Zeng, Z., Mao, A. and Chen, S. (2014), 'Homologous overexpression of alkyl hydroperoxide reductase subunit C (ahpC) protects Bifidobacterium longum strain NCC2705 from oxidative stress', *Research in Microbiology* **165**(7), 581–589. Type: Journal Article.
URL: *https://www.sciencedirect.com/science/article/pii/S0923250814000886*

Zuo, F., Yu, R., Xiao, M., Khaskheli, G. B., Sun, X., Ma, H., Ren, F., Zhang, B. and Chen, S. (2018), 'Transcriptomic analysis of Bifidobacterium longum subsp. longum BBMN68 in response to oxidative shock', *Scientific Reports* **8**(1), 17085. Type: Journal Article.
URL: *https://doi.org/10.1038/s41598-018-35286-7*