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Enhanced stress resistance of *Bifidobacterium breve* NRBB57 by induction of stress proteins at near-zero growth rates

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RESEARCH ARTICLE

Abstract

Bifidobacterium breve is a common habitant of the human gut and is used as probiotic in functional foods. *B. breve* has to cope with multiple stress conditions encountered during processing and passage through the human gut, including high temperature, low pH and exposure to oxygen. Additionally, during industrial processing and in the gut, *B. breve* could encounter nutrient limitation resulting in reduced growth rates that can trigger adaptive stress responses. For this reason, it is important to develop culture methods that elicit resistance to multiple stresses (robustness) encountered by the bacteria. To investigate the impact of caloric restriction on robustness of the probiotic *B. breve* NRBB57, this strain was grown in lactose-limited chemostat cultures and in retentostat for 21 days, at growth rates ranging from 0.4 h^{-1} to 0.00081 h^{-1}. Proteomes of cells harvested at different growth rates were correlated to acid, hydrogen peroxide and heat stress survival capacity. Comparative proteome analysis showed that retentostat-grown cells had significantly increased abundance of a variety of stress proteins involved in protein quality maintenance and DNA repair (DnaJ, Hsp90, FtsH, ClpB, ClpP1, ClpC, GroES, RuvB, RecA), as well as proteins involved in oxidative stress defence (peroxiredoxin, ferredoxin, thioredoxin peroxidase, glutaredoxin and thioredoxin reductase). Exposure to three different stress conditions, 45 °C, pH 3, and 10 mM H_2O_2, showed highest stress resistance of retentostat cells sampled at week 2 and week 3 grown at 0.0018 and 0.00081 h^{-1}. Our findings show that cultivation at near-zero growth rates induces higher abundance of stress defence proteins contributing to the robustness of *B. breve* NRBB57, thereby offering an approach that may support its production and functionality.

Keywords: probiotic, retentostat, stress response, proteomics, robustness

1. Introduction

During the last decades, the study of functional foods containing probiotic bacteria has become an important area of research in academia and in food industry (Mills *et al.*, 2011; Tripathi and Giri, 2014). Next to studies on ecophysiology including survival capacity and fermentation capacity, also aspects like functionality and potential health effects have gained ample attention. The FAO/WHO definition of a probiotic – 'live microorganisms that when administered in adequate amounts can confer a health benefit on the host' (FAO/WHO, 2006), was reinforced in an expert consensus document as relevant and sufficiently accommodating for current and anticipated applications (Hill *et al.*, 2014). Among the most widely investigated species of bacteria as active ingredient of functional foods are species in the genus *Bifidobacterium* (Amund *et al.*, 2014). These anaerobic Gram-positive (branched) rods are among the first colonisers of the human gastrointestinal tract right after birth (Kumar *et al.*, 2020; Milani *et al.*, 2017). Several studies have attributed numerous probiotic properties to these bacteria, including alleviation of disturbances in colonic transit (Muñoz *et al.*, 2011; Tabbers *et al.*, 2011), stimulation of the immune system (Arunachalam *et al.*, 2000; Makino *et al.*, 2013), competitive exclusion of pathogens by attachment on epithelial cells (Gueimonde *et al.*, 2005; Hidalgo-Cantabrana *et al.*, 2017; O'Callaghan and van Sinderen, 2016) and prevention of depression symptoms (Yang *et al.*, 2017). Within the *Bifidobacterium* genus, the species *Bifidobacterium breve,*

which was first isolated from faeces of a breast-fed newborn, is used in dairy fermentations and in infant formulas, and in a range of probiotic applications (Bozzi Cionci *et al.*, 2018).

However, the use of probiotic bacteria like *B. breve* as active ingredient represents a big challenge for food producers. Probiotic products should contain an appropriate concentration of viable bacteria to be considered suitable for consumption (Talwalkar and Kailasapathy, 2004; Vinderola *et al.*, 2011). This means that food producers have to ensure that their final products can maintain the functionality of the probiotic bacteria until the time of consumption. Functionality is often compromised because probiotic bacteria have to endure several stressful conditions during the production processes, including exposure to oxygen and exposure to high temperatures (Gaucher *et al.*, 2019; Talwalkar and Kailasapathy, 2004). The damage caused by heat stress causes cell permeability leading to leakage and damage of intracellular components (Meng *et al.*, 2008). Likewise, anaerobic bacteria are greatly affected by oxidative stress when reactive oxygen species are formed leading to damage of DNA, proteins and lipids, ultimately causing bacterial death (Feng and Wang, 2020). Therefore, bacterial robustness has become an important parameter when it comes to developing new probiotic products. Consequently, there is an interest in non-GMO technologies that can enhance bacterial robustness.

Recent studies describe the use of the so-called retentostat cultivation to determine the impact of near-zero growth rates on microbial physiology including activation of stress resistance (Ercan *et al.*, 2022; Goffin *et al.*, 2010; Vos *et al.*, 2016). Retentostat cultivation is a variation on the classical chemostat cultivation and ensures complete retention of cells by inserting a cross-flow filter in the effluent line. In this way biomass accumulates in the bioreactor, reducing the energy and nutrient supply per cell and thus progressively decreasing the growth rate of individual cells which eventually approaches zero. As a consequence, all energy and nutrients supplied to the bioreactor will be directed to cellular processes involved in maintenance (Van Mastrigt *et al.*, 2019a).

In a previous study, using *B. breve* NRBB57 as a model, we determined the impact of near-zero growth rates in a lactose-limited retentostat on cellular parameters including culturability, viability, and metabolic pathway activation using a combined proteomics and metabolomics approach (Ortiz Camargo *et al.*, 2022).The aim of the current study is to extend this analysis by determining the impact of imposed near-zero growth rates on *B. breve* NRBB57 stress defence proteomes and to identify correlations with stress resistance following exposure to (lethal) heat, low pH and hydrogen peroxide stress.

2. Materials and methods

Bacterial strain and culture conditions

B. breve NRBB57 was used throughout the study, and stock suspensions were kept at -80 °C in growth medium supplemented with glycerol (30%, v/v) until its use. The glycerol stocks were streaked on TOS-propionate agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 48 h in anaerobic jars (Advanced Instruments, Norwood, MA, USA) with anaerobic gas-generating sachets (Oxoid™ AnaeroGen™; ThermoFisher Scientific, Waltham, MA, USA). The overnight cultures were made by inoculating a single colony of *B. breve* NRBB57 in 10 ml of TOS-propionate broth (Merck). The TOS-propionate broth) was made according to manufacturer's instructions and passed through a filter paper grade 520B (Whatman, Maidstone, UK) to separate the agar from the broth. Lastly, overnight cultures were incubated at 37 °C for 20 h in anaerobic jars (Advanced Instruments) with anaerobic gas-generating sachets (Oxoid™ AnaeroGen™) and subsequently used to inoculate chemostat cultures.

Bifidobacterium breve culture media

In the chemostat and retentostat cultivations, a culture medium was used containing per kg: 5.4 g lactose.H_2O as carbon source, 10 g bacto-tryptone, 2.7 g KH_2PO_4, 0.25 g $MgSO_4.7H_20$, 0.5 g L-cysteine-HCl, 1 g yeast extract, 0.002 g D-biotin, 0.0005 g cyanocobalamin, 0.01 g Ca-(D+) panthothenate, 0.005 g nicotinic acid, 0.005 g *p*-aminobenzoic acid, 0.005 g thiamin-HCl, 0.008 g pyridoxamine-HCl, 0.001 g riboflavin, 0.01 g adenine, 0.01 g xanthine, 0.01 g guanine, 0.01 g uracil, 0.05 g $MgCl_2.6H_2O$, 0.02 g $MnSO_4.H_2O$, 0.005 g $ZnSO_4.7H_2O$, 0.0025 g $CuSO_4.5H_2O$, 0.005 g $FeCl_2.4H_2O$ and 0.01 g $CaCl_2.2H_20$. Once the components were weighted, they were properly mixed with deionised water and after all compounds were dissolved, the pH was adjusted to 6.5 by adding 10 M NaOH. Finally, the medium was sterilised by passing it through a filter with an average pore size of 0.2 µm (Sartopore 2 KLG; Sartorious Stedim, Göttingen, Germany).

Chemostat cultivation

B. breve NRBB57 was cultivated in a chemostat at dilution rates of 0.4, 0.25, 0.12 and 0.025 h^{-1} in 0.5 l bioreactors (Multifors, Infors HT, Bottmingen, Switzerland). Briefly, the bioreactor was inoculated with 1% (v/v) overnight culture previously made in TOS-propionate broth (Merck). Bacteria were grown until the end of the exponential phase when the supply of fresh medium was turned on to start the chemostat mode at the selected dilution rates. The stirring speed was set at 300 rpm, the temperature at 37 °C, and the pH was set at 6.5 which remained constant during the fermentation run by automatic addition of 5 M NaOH.

Anaerobic environment was achieved by flushing the headspace with gas composed by 5% CO_2, 5% H_2, 90% N_2 at a rate of 0.06 l/min. Samples were taken after steady states were reached, which was considered to be after a minimum of 5 volume changes.

Retentostat cultivation

B. breve NRBB57 was cultivated in retentostat mode using 1 l bioreactors (Multifors, Infors HT, Switzerland) which were operated in biological duplicates. The bioreactors were inoculated with 1% (v/v) overnight culture made in TOS-propionate broth. Bacteria were grown until the end of the exponential phase when fresh medium supply was turned on to start the chemostat mode at a dilution rate of 0.025 h^{-1}. Once the chemostat reached steady state, a polyether sulfone crossflow filter (0.2 µm; Spectrum Laboratories, Rancho Dominguez, CA, USA) was connected to an outer loop in the effluent line to begin the retentostat mode. Temperature (37 °C), pH (6.5), stirring speed (300 rpm) and the anaerobic environment were kept constant as previously described for the chemostat cultures. Retentostat cultures were run for approximately three weeks. Samples were taken once a week.

Proteome analysis

Proteomes were analysed using samples collected from 2 biological replicates of chemostats at dilution rates of 0.4, 0.25, 0.12 and 0.025 h^{-1} (which corresponds to time 0 of the retentostats), and from 2 biological replicates of the retentostat after 1, 2 and 3 weeks of cultivation. These samples were centrifuged at 13,000×*g* for 5 min and pellets were frozen at -80 °C until further use. Initially, cells were lysed by bead beating in a FastPrep-24TM 5G instrument (MP Biomedicals, Irvine, CA, USA) for 6 times 30 s at 6.5 m/s with cooling after every bead step. The protein concentration was assessed using Pierce BCA protein assay (ThermoFisher Scientific, Waltham, MA, USA) and protein digestion was done overnight. Samples were cleaned and then analysed by Nano liquid chromatography-high-resolution mass spectrometry (nano-LC-HRMS/MS) as previously described (Meiring *et al.*, 2002). The acquired spectra were analysed using Thermo Proteome Discoverer in combination with Mascot (ThermoFisher Scientific). The reference database comprised of protein sequences from *B. breve* NRBB57 from Uniprot and contaminant proteins which were discriminated. Relative protein quantification was performed by Proteome Discoverer based on peptide intensity signals using default settings. Finally, to analyse the data, we determined which proteins significantly differ between growth rates. For this analysis, the abundances of the proteins were plotted versus the growth rate and linear regression was used to find the slopes of the line and how significant this slope differed from 0 (*P*-value) in R (v3.63). Significant differences were considered when the *P*-value of the slope was <0.05.

Stress performance analysis

In addition to proteomics analysis, part of the samples taken were used to quantify stress survival capacity of the respective chemostat (t=0) and retentostat cells after 1, 2 and 3 weeks of cultivation. The bacterial suspensions were exposed to the following conditions: (1) 2 h at pH 3; (2) 2 h at 45 °C; and (3) 1 h in 10 mM of hydrogen peroxide. For stress survival assays, 100 µl of the sample was re-suspended in 900 µl of fresh medium in Eppendorf tubes depending on the stress condition with adjusted pH 3, pre-heated at 45 °C, or with 10 mM of hydrogen peroxide. Samples of 30 µl of each tube were taken at time 0 and at the end of the of the stress exposure. These samples were serially diluted in peptone physiological salt solution (PPS; Tritium Microbiology, Eindhoven, the Netherlands) and appropriate dilutions were plated on TOS agar (Merck). The plates were then incubated anaerobically at 37 °C for 48-72 h, after which the colonies were counted. Stress exposure experiments were carried out in aerobic conditions to mimic industrial conditions where oxygen can be present during certain productions steps. Percentage of survival was calculated considering the number of cells at time 0 set at 100%. To determine possible significant differences (*P*-value <0.05) between the survival percentages for the different conditions, one way ANOVA was carried out in GraphPad 9.0.1 (La Jolla, CA, USA).

Stress proteins abundance vs survival analysis

To determine which proteins have significant effects of the stress survival, abundances of all stress proteins were correlated to survival to the above described stresses using R (v3.63). Linear regression was used to calculate the slope and its significance. *P*-values below <0.05 were considered significant.

3. Results

Increased abundance of proteins involved in stress response

A comparative proteomics analysis was performed using *B. breve* NRBB57 cells obtained from anaerobic chemostat and retentostat cultivations at all tested growth rates in a medium containing lactose as growth-limiting substrate. Our analysis shows expression of 20 proteins associated with stress response in chemostat and retentostat cells, with three proteins, ClpP2, GroEL and RuvA, showing no significant differential expression, four proteins, DnaK, GrpE, RecN, and glutaredoxin, showing higher abundance in chemostat cells, and 13 proteins are up-regulated in retentostat cells at near-zero growth rates, ClpB, ClpC, ClpP1, GroES, Hsp90, FtsH, DnaJ RuvB, RecA, ferredoxin, peroxidoxin, thioredoxin reductase, and thioredoxin peroxidase (Figure 1A). The upregulated proteins have been

grouped according to putative roles in resistance to lethal heat, low pH and oxidative stress (De Dea Lindner *et al.*, 2007; Feng and Wang, 2020; Zomer *et al.*, 2009) (Figure 1B). It should be noted that most of the 20 identified proteins contribute to defence against a range of stresses (Ling *et al.*, 2018; Zomer and Van Sinderen, 2010).

Stress tolerance and correlation to stress proteins

As follow-up of the previous results, we determined if the apparent activation of stress defence proteins in retentostat cells of *B. breve* NRBB57 confers an augmented robustness. Therefore, we determined survival of retentostat cultivated cells of *B. breve* NRBB57 upon exposure to lethal heat stress (2 h at 45 °C), low pH (2 h at pH 3), and oxidative stress (1 h in 10 mM H_2O_2) (Figure 2). In this analysis, samples were withdrawn from the bioreactor simultaneous with the samples taken for proteomics analysis. Furthermore, the performance of retentostat cells was compared to that of chemostat cells grown at 0.025 h^{-1} (t=0 sample) preceeding the shift to the retentostat growth mode.

Following heat stress exposure, control cells cultivated in the chemostat had the lowest percentage of survival (5.8%), while retentostat-grown cells showed enhanced performance. The highest survival (70%) was found for cells sampled after 3 weeks (0.0008 h^{-1}), although, differences were not significant. Exposure to low pH resulted again in lowest survival of chemostat-grown cells (8.8%), while retentostat-grown cells showed enhanced performance with highest survival (40.8%) of cells sampled after three weeks (0.0008 h^{-1}). The retentostat cells also showed higher survival efficacy following exposure to oxidative stress, with an approximate 6.000-fold better performance of retentostat cells harvested after two weeks (0.0018 h^{-1}) compared to chemostat cells grown at 0.025 h^{-1} (0.25% versus 0.00004% survival).

A correlation analysis was performed to investigate the relationship between the relative abundance of specific stress defence proteins and survival of *B. breve* NRBB57 exposed to lethal heat, low pH and oxidative stress conditions. From the tests carried out, only the ones with a p-value <0.05 were considered significant, and this resulted in 15 significant correlations with the stress tolerance of *B. breve* (Figure 3). The highest number of correlations were found for low pH and high temperature exposure. In these

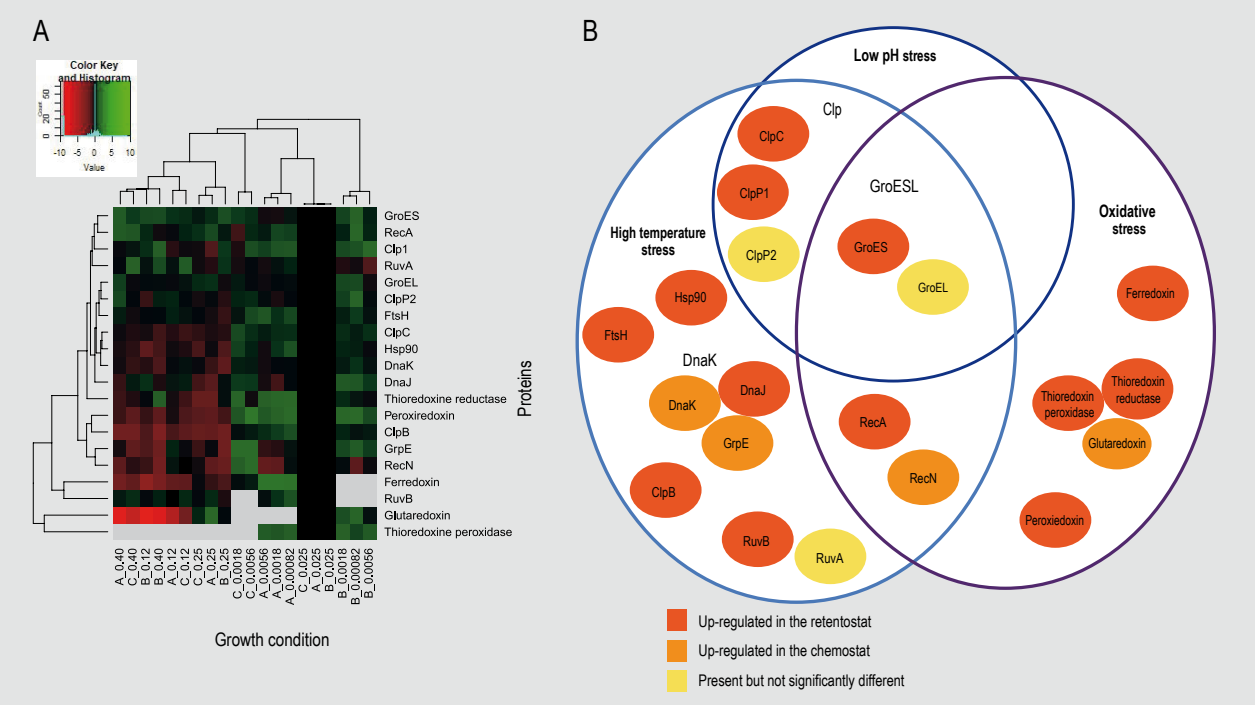

Figure 1. (A) Heat map of the proteins of *Bifidobacterium breve* NRBB57 involved in stress response with abundances at different growth rates in chemostat (0.4, 0.25, 0.12 and 0.025 h^{-1}) and retentostat cultivation (0.0055, 0.0018, 0.0008 h^{-1}). A and B each correspond to one biological replicate of every growth rate. Values are given as log_2 ratio compared to the growth rate of 0.025 h^{-1}. Green cells represent upregulated proteins, red cells represent downregulated proteins. Grey cells represent proteins that could not be quantified. (B) Classification of the proteins with putative roles in high temperature, low pH and oxidative stress resistance. Dark orange represents the proteins that were up-regulated in the retentostat, light orange the proteins that were up-regulated in the chemostat at lower growth rates, and in yellow the proteins that were present but show no significantly different abundance compared to that of chemostat cells grown at 0.025 h^{-1}.

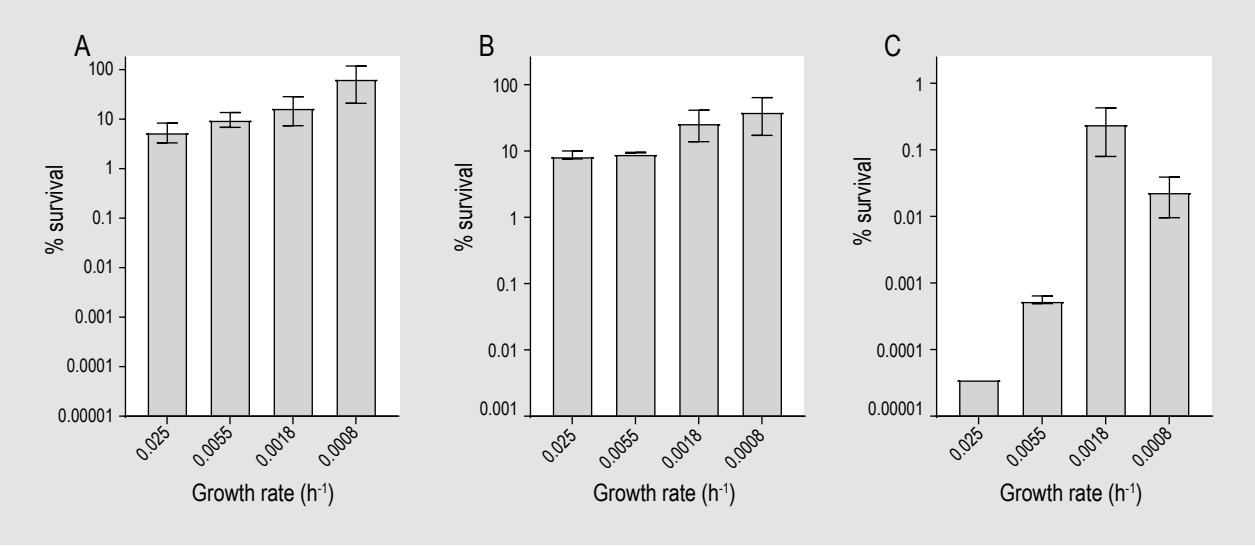

Figure 2. Relative survival of chemostat (μ=0.025 h^{-1}) and retentostat (μ=0.005 h^{-1} to μ=0.00082 h^{-1}) cultivated cells of *Bifidobacterium breve* NRBB57 after exposure for 2 h at 45 °C (A), after 2 h exposure at pH 3 (B), and after 1 h exposure to 10 mM of H_2O_2 (C). Bars represent the average of measurements of 2 independent replicates for every growth rate.

2 conditions high stress tolerance of retentostat grown cells compared to chemostat grown cells was observed.

Proteins correlating to low pH and high temperature resistance include: GroES, GroEL, and Clp2 involved in maintenance of protein quality, DnaK, RecA, RuvB, involved in DNA replication and damage repair, and glutaredoxin and peroxiredoxin which are normally involved in oxidative stress resistance (Maleki *et al.*, 2016; Papadimitriou *et al.*, 2016; Perkins *et al.*, 2015; Shiba *et al.*, 1993; Ventura *et al.*, 2004). All other proteins did not show significant correlations with the tolerance to the different stress conditions.

4. Discussion

The present study shows that cultivation of *Bifidobacterium breve* NRBB57 at near-zero growth rates in the retentostat results in increased expression of many stress defence proteins that correlated with enhanced survival capacity of lethal heat, low pH and oxidative stress.

Stress proteins with higher abundances at lower growth rates

High temperature and oxidative stress tolerance in bifidobacteria are of great importance when it comes to industrial production processes. For instance, during the production of probiotic formulas, bacteria go through extreme conditions involving high temperatures (e.g. spray drying) and the presence of oxygen (e.g. mixing and packaging). Furthermore, probiotic bacteria also encounter stress inducing conditions in the GI tract following consumption including exposure to low pH during stomach passage. Therefore, it is of particular interest to prime cells for the induction of proteins involved in protection of the cells towards such harsh conditions.

Our proteomics analysis showed expression of 20 proteins related to stress response in chemostat and retentostat cells, with three proteins (ClpP2, GroEL and RuvA) showing no significant differential expression, four showing higher abundance in chemostat cells (DnaK, GrpE, RecN, glutaredoxin), and 13 proteins showing higher abundance in retentostat cells at near-zero growth rates, namely ClpB, ClpC, ClpP1, GroES, Hsp90, FtsH, DnaJ RuvB, RecA, ferredoxin, peroxidoxin, thioredoxin reductase, and thioredoxin peroxidase. Most of these proteins function as chaperones which play a crucial role in maintenance of cell viability, homeostasis and culturability by preventing formation and accumulation of misfolded and/or aggregated (membrane) proteins (DnaJ, Hsp90, ClpB, ClpC, ClpC1, GroES, and FtsH) and repair of DNA damage (RecA, RecN, RuvA, RucB, DnaK) (Zomer *et al.*, 2009; Zomer and van Sinderen, 2010). The protein repair group of chaperons found in our study, includes proteins reported to be part of the stress defence in response to exposure to heat, low pH and oxidative stress conditions in a variety of bifidobacteria including *B. breve* UCC2003 (Farr and Kogoma, 1991; Ventura *et al.*, 2004, 2005a,b,c). Based on *in silico* analysis and gene expression studies in *B. breve* UCC2003, an interacting regulatory network model for stress response, composed of HspR, ClgR and HrcA, was previously proposed (Zomer *et al.*, 2009; Zomer and van Sinderen, 2010). Our proteomics study now provides evidence for increased expression of stress defence proteins, including Clp chaperones involved in protein quality homeostasis (Schelin *et al.*, 2002; Ventura *et al.*,

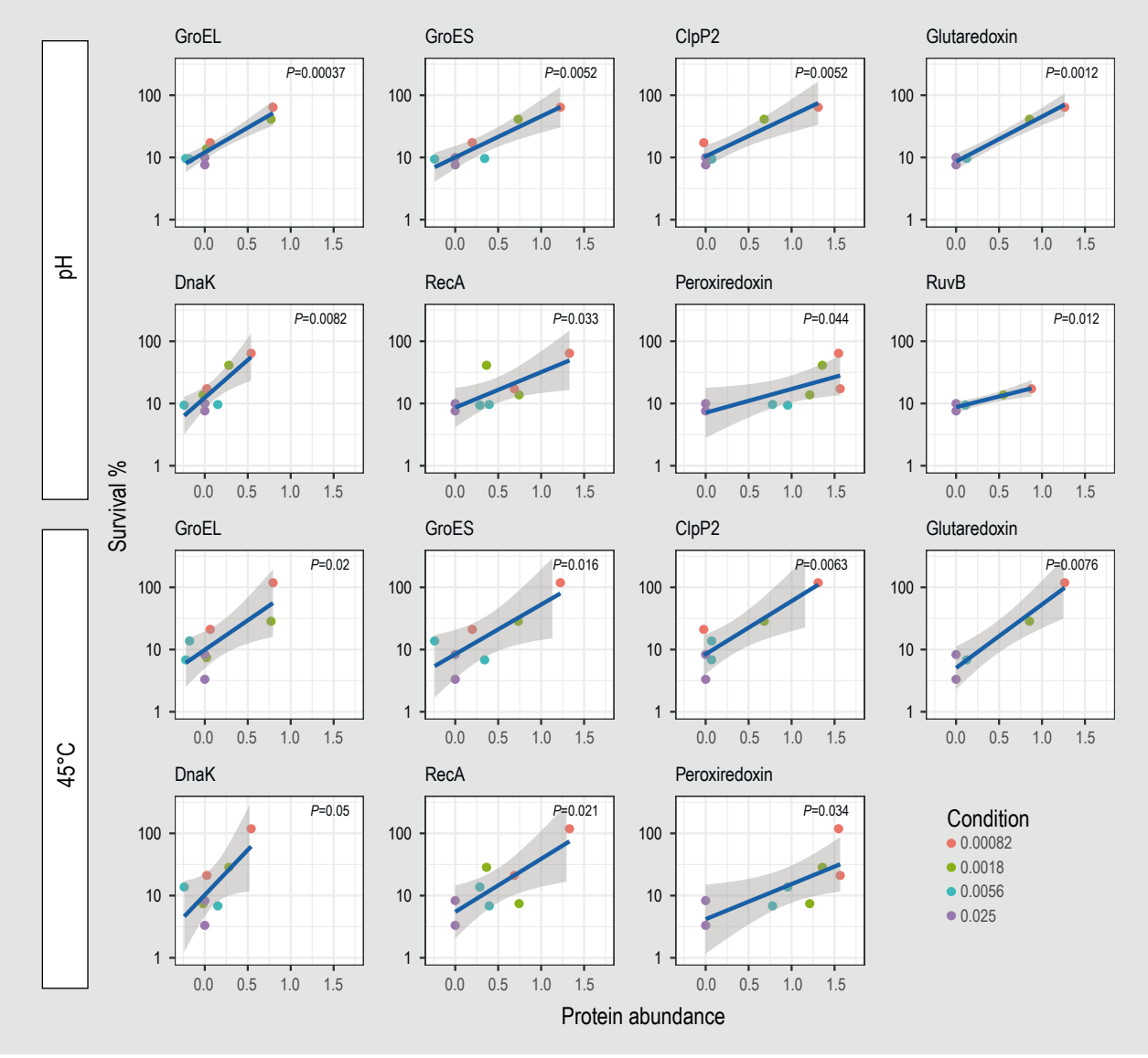

Figure 3. Percentage of survival of *Bifidobacterium breve* NRBB57 after exposure to pH 3 and 45 °C for 2 h against the abundances of the up-regulated stress proteins. Only up-regulated stress proteins that showed a significant correlation (*P*<0.05) with heat and low pH stress tolerance of *B. breve* NRBB57 are shown. Different coloured data points represent the biological replicates of each growth rate while grey areas are the confidence intervals.

2005a) and proteins involved in DNA protection and repair, including RecA, involved in transcription activation of SOS regulon members, *ruvA* and *ruvB* (Cox, 2007; Podlesek and Zgur Bertok, 2020; Shiba *et al.*, 1993; Zomer and Van Sinderen, 2010). Lastly, four proteins are upregulated in retentostat cells that have a specialised function in oxidative stress response: thioredoxin peroxidase, thioredoxin reductase, ferredoxin and peroxiredoxin. The thioredoxins have several roles in the stress response: reduction of peroxides, protein folding, catalysis of biological thiol-disulphide exchange reactions and reduction of oxidised redox sensors and helping in the transcription of genes involved in physiological responses against oxidative stress (Gonzalez *et al.*, 2020; Sun *et al.*, 2019). Peroxiredoxin and ferredoxin scavenge peroxides and other toxic forms of oxygen. Finally, glutaredoxin, which main role is as electron donor contributing to cytoplasmic redox homeostasis (Feng and Wang, 2020; Grant, 2001). A recent study described the creation of a large Tn*5* transposon mutant library of the commensal *B. breve* UCC2003 that was further characterised by means of a Transposon Directed Insertion Sequencing (TraDIS) approach (Ruiz *et al.*, 2013). This analysis revealed a set of genes essential for growth of this strain under *in vitro* conditions. The identified set of essential genes also includes functions, such as glutaredoxin production and thioredoxin reductase and these genes are present in the core genome of all tested species of the genus *Bifidobacterium* spp. The latter highlights a high

degree of conservation of essential genes at the genus level (Ruiz *et al.*, 2017).

Notably, bifidobacteria lack a typical general stress sigma factor such as RpoS found in *Proteobacteria* and Sigma B present in many *Firmicutes*, that activate stress defence upon exposure to a wide range of stress conditions (Dodd and Aldsworth, 2002; van Schaik and Abee, 2005). In addition to previously proposed roles for transcriptional regulators in *B. breve* UCC2003 that activate stress defence in selected stress conditions conceivably by loss of repressor function (Zomer *et al.*, 2009; Zomer and Van Sinderen, 2010), our current study provides evidence for activation of a multiplicity of stress defence proteins in retentostat cells at near-zero growth rates, conceivably linked to the induction of the stringent response in *B. breve* NRBB57 under these extreme nutrient-limited conditions (Ortiz Camargo *et al.*, 2022). The stringent response is known to be induced by nutrient deprivation, and is mediated by the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Irving *et al.*, 2021), and (indirectly) controls expression of stress defence proteins, such as DnaK, GroESL, and ClpB (Carneiro *et al.*, 2011; Schäfer *et al.*, 2020), and oxidative stress response proteins such as thioredoxin and glutaredoxin (Khakimova *et al.*, 2013; Ma *et al.*, 2019). Activation of genes involved in stringent response was described for a range of bacteria including acid-stressed *B. longum* (Jin *et al.*, 2012; Schell *et al.*, 2002). In addition, using transcriptomics, ^{13}C-labeling and metabolomics (Schofield *et al.*, 2018) showed that the stringent response determined the ability of the gut commensal *Bacteroides thetaiotaomicron* to survive starvation and to persist in the gut. Combining all information suggests an important role of ppGpp-mediated activation of stringent response and subsequent metabolic shifts and activation of multiple stress defences in retentostat grown *B. breve* cells at near-zero growth rates.

Stress tolerance to high temperatures, low pH and oxidative stress

To corroborate a possible stress pre-adaptation in retentostat cultivated bifidobacteria at near-zero growth rates, we tested the tolerance of these bacteria to three different lethal stress conditions. Retentostat cells sampled after 2 and 3 weeks (growth rates 0.0018 h^{-1} and 0.0008 h^{-1}, respectively) showed higher survival compared to chemostat cells when exposed to lethal heat stress (maximum 70 versus 5.8% survival) and to low pH stress (maximum 40.8 versus 8.8%) and oxidative stress (maximum 0.25 versus 0.00004%). Despite low survival percentage of retentostat cells in the tested severe oxidative stress conditions (60 min exposure to 20 mM hydrogen peroxide), their highest survival efficacy is approximately 6.000-fold higher than that of chemostat cells, indicating activation of efficient stress defence, conceivably due to activation of thioredoxin peroxidase, thioredoxin reductase, ferredoxin and peroxiredoxin, described above. These results indicate that retentostat grown *B. breve* NRBB57 cells can activate a multiple stress defence, with a prominent relative increase in oxidative stress survival capacity. Comparing *B. breve* stress survival capacity with that of *Lactococcus lactis* and *Lactiplantibacillus plantarum* retentostat cells exposed to similar stresses, shows higher stress tolerance of the latter species (Ercan *et al.*, 2022). It should be noted that in the current experimental set up, no additional measures were taken to prevent exposure to oxygen in the stress survival experiments. This was done to mimic conditions encountered during processing in factory environments. Since bifidobacteria are strict anaerobic bacteria, it is conceivable that exposure to air/oxygen imposes additional stress on the cells, and this may explain why despite the increased abundances of stress defence proteins in *B. breve* NRBB57 retentostat cells, survival capacity appears somewhat lower compared to that of facultative anaerobic lactic acid bacteria. Since retentostat cultivations involve long run times, up to two to three weeks, other alternatives could be explored to minimise the cultivation times. One of these alternatives is the so-called partial cell-recycling system (Van Mastrigt *et al.*, 2019b). In this approach, cells are partially fed back to the bioreactor, which allows cultivation of bacteria at near-zero growth rates in a shorter time of just few days. Translation of the current findings to industrial applications requires further study.

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Conflict of interest

RSB, KBA and JK are employees of Danone Nutricia Research.

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