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RESEARCH ARTICLE

Enhancing survival of *Bifidobacterium* spp. in yoghurt through oxidative stress adaptation

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Background:
Bifidobacterium *spp. are widely recognised probiotic bacteria with well-documented health benefits associated with their incorporation into foods. Despite these benefits, maintaining their viability in oxygen-rich environments like yoghurt poses substantial challenges.*

Aim:
This study examined the impact of oxidative stress adaptation on the viability, fermentation characteristics and physicochemical properties of Bifidobacterium *spp. during yoghurt production and shelf-life at 4°C.*

Methods:
Oxidative (H_2O_2) stress-adapted and unadapted strains of Bifidobacterium bifidum, B. breve *and* B. animalis *subsp.* animalis, *6–7 log cfu/mL, were incorporated during yoghurt fermentation. Two independent yoghurt batches were produced for each treatment combination, with two replicates analysed per batch. Viability (via plate counts and PMAxx-qPCR) and physicochemical properties were monitored over 28 days at 4°C.*

Major Findings:
Stress adaptation enhanced B. bifidum *survival during fermentation by 1.0 log cfu/g compared with unadapted strains (*$P < 0.0001$*), with* B. breve *showing similar improvement. Although adaptation initially improved survival, this advantage diminished throughout shelf-life in both species.* B. animalis *remained stable. The plate count method showed a decline of* B. bifidum *and* B. breve *populations, while the PMAxx-qPCR method detected a significantly higher level of viable cells (*$P < 0.05$*) in the yoghurt.*

Scientific Implications:
Oxidative stress adaptation may enhance Bifidobacterium *spp. viability and the functional value of probiotic yoghurt.*

Keywords *Bifidobacterium* spp., Yoghurt, Oxidative stress adaptation, Viability, PMAxx-qPCR, Probiotic.

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INTRODUCTION

The increasing consumer focus on health-beneficial products has stimulated research into functional foods containing probiotic microorganisms (Grujović *et al*. 2025). Yoghurt and other dairy matrices are widely used as delivery vehicles for these beneficial bacteria because they can provide the high cell density required for efficacy (Zhang *et al*. 2023). Scientific guidelines generally recommend that at least 6–7 log cfu of viable probiotics per millilitre reach the intestine to confer health benefits (Neffe-Skocińska *et al*. 2018). Given that the typical yoghurt serving in South Africa is approximately 200 g, this translates into a target of at least 8 log cfu per serving to ensure that consumers ingest a therapeutically relevant dose during yoghurt consumption (Sibanda *et al*. 2024).

Within the probiotic category, *Bifidobacterium* spp. are recognised for providing substantial health advantages when incorporated into food

products (Idf and Federation 2015; Nyanzi *et al*. 2021; Sibanda *et al*. 2024). Nevertheless, integrating these organisms into probiotic formulations poses significant technological difficulties. Their obligate anaerobic nature renders *Bifidobacterium* spp. vulnerable to oxygen exposure, creating substantial viability challenges throughout yoghurt production (Kawasaki *et al*. 2018; Schöpping *et al*. 2022; Sibanda *et al*. 2024). As a result, the counts of *Bifidobacterium* spp. often fall short of the recommended therapeutic viability level throughout shelf-life, reducing their potential health benefits. For technological reasons, most dairy probiotic starter cultures incorporate the aerotolerant *B. animalis* subsp. *lactis*, with proven enhanced survivability during yoghurt manufacturing (He *et al*. 2023). However, many other species, such as *B. bifidum, B. breve* and *B. longum*, are important for a healthy human gut (Chen *et al*. 2021). Therefore, while these species are anaerobes, their inclusion in probiotic dairy products could offer a solution to treating gut dysbiosis (Bocchio *et al*. 2025).

Stress adaptation is the process by which microorganisms develop resistance to environmental stressors through repeated exposure to sublethal levels across multiple generations, transforming from initially susceptible organisms into stress-tolerant variants (Schöpping *et al*. 2023). This process offers promise for enhancing *Bifidobacterium* spp. resilience to oxidative conditions, enhancing their viability both during yoghurt production and shelf-life (Schöpping *et al*. 2022; Sibanda *et al*. 2024). The adaptation treatment induces physiological and genetic responses that enhance bacterial resilience, increasing their chances of survival during processing and shelf-life (Settachaimongkon *et al*. 2015; Schöpping *et al*. 2022). Successive cultivation of sublethally exposed cells under lethal conditions can reinforce adaptive responses, producing strains with stable, enhanced stress resistance. However, how oxidative stress adaptation affects the survival dynamics of *Bifidobacterium* spp. during yoghurt storage remains insufficiently explored. This process offers promise for enhancing *Bifidobacterium* spp. resilience to oxidative conditions, enhancing their viability both during yoghurt production and shelf-life (Oberg *et al*. 2013; Jin *et al*. 2015; Wei *et al*. 2019).

This study aimed to evaluate oxidative stress adaptation as a strategy to enhance the survival and maintain the therapeutically appropriate cell concentrations of oxygen-sensitive *Bifidobacterium* spp. (*B. bifidum, B. breve* and *B. animalis*) throughout yoghurt production and refrigerated shelf-life at 4°C.

MATERIALS AND METHODS

Bifidobacterium cultures

Three *Bifidobacterium* species were obtained from Microbiologics (MN, USA): *B. bifidum* ATCC® 11863™, *B. breve* ATCC® 15700™ and *B. animalis* subsp. *animalis* ATCC® 25527™ (Microbiologics, MN, USA). Lyophilised cultures were reconstituted in 10 mL of sterile MRS-C broth [MRS broth (De Man *et al*. 1960) supplemented with 0.05% L-cysteine (w/v)] and incubated anaerobically at 37°C for 24 h. Anaerobic conditions were maintained using anaerobic jars equipped with gas-generating sachets (AnaeroGen™, Oxoid Ltd, Basingstoke, UK). *Bifidobacterium* spp. were cryopreserved in 25% sterile glycerol (v/v) at −80°C using cryotubes or cryobeads. Prior to experimentation, cultures were resuscitated during 48-h anaerobic incubation in MRS-C broth at 37°C, unless otherwise mentioned.

Oxidative stress treatments

Hydrogen peroxide MIC

Hydrogen peroxide (H_2O_2) minimum inhibitory concentrations (MIC) for each *Bifidobacterium* spp. were established following the broth microdilution protocol outlined by Ibraheim *et al*. (2020). Bacterial colonies grown on MRS-C agar at 37°C were resuspended in peptone buffered saline (PBS, pH 7.3) containing 0.05% (w/v) L-cysteine to achieve a turbidity matching the 0.5 McFarland standard (approximately 8 log cfu/mL).

Standardised inoculums (2 µL) were added to 96-well microtitre plates containing 200 µL of anoxic MRS broth with H_2O_2 concentrations ranging from 0 to 3.2 mM. Prior to inoculation, the plates were placed in an anaerobic jar with gas-generating sachets for 24 h to ensure an anoxic environment. Plates were incubated at 37°C for 48 h under anaerobic conditions. The MIC was determined as the minimum H_2O_2 concentration that completely inhibited growth, assessed by measuring OD_{600} readings (FLUOstar® Omega Microplate reader), visual inspection of turbidity and standard plate counting (SPC). The obtained MIC values were used in the subsequent adaptation experiments. Following H_2O_2 exposure, multiple colonies from at least three dilution levels were pooled across replicates to ensure genetic homogeneity of adapted populations. Strain identity was confirmed by MALDI-TOF MS. Consequently, sublethal H_2O_2 concentrations were established at 0.4 mM for *B. bifidum* and *B. breve* and 0.8 mM for *B. animalis*, while a lethal concentration of 1 mM H_2O_2 was used for all species.

Oxidative stress adaptation treatments

A two-stage H_2O_2 treatment was applied to *B. bifidum*, *B. breve* and *B. animalis* cultures. Mid-log phase cultures, collected after 6 h of growth, were subjected to a 48-h incubation at 37°C with sublethal concentrations, as specified previously. Subsequently, viable cell populations were determined and isolated through spread-plating on MRS-C agar, incubating at 37°C for 48 h. Resulting colonies, representing the sublethal H_2O_2-treated cells, were cryopreserved for subsequent treatment phases.

To apply the lethal stress, previously exposed cells were grown on MRS-C agar under anaerobic conditions for 24 h, then subjected to 1 mM H_2O_2 in anoxic MRS broth at 37°C for 30 min. Surviving cells were subsequently recovered by plating

on MRS-C agar and incubating anaerobically at 37°C for 48 h. Colonies obtained from the initial round of lethal H_2O_2 exposure underwent two further cycles of identical lethal treatment. The resulting third-generation colonies were stored at −80°C as the final adapted strains. Nonadapted cultures were used as control strains throughout the study.

Bifidobacterium culture preparation for yoghurt production

Stock cultures of unadapted and oxidative stress-adapted *Bifidobacterium* spp. were grown in MRS-C broth under anaerobic conditions at 37°C for 48 h. The activated cultures were then subcultured at 1% (v/v) into 50 mL fresh MRS-C broth and incubated anaerobically at 37°C for an additional 48 h. Prior to yoghurt inoculation, the subcultures broth was subjected to centrifugation (5000 × *g*; 10 min) using a Hermle Z 366 K centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). The resulting cell pellet was resuspended in 10 mL of pasteurised milk to prepare the inoculum for yoghurt fermentation.

Yoghurt production and shelf-life analysis

Whole bovine milk containing 3% fat, 8% fat-free solids, 11% total solids was sourced from the University of Pretoria Experimental Farm (Pretoria, South Africa). Milk portions (250 mL) were mixed with 1.5% stabiliser (w/v) (acetylated di-starch adipate, E1422) and allowed to hydrate for 30 min before pasteurisation (90°C; 10 min). Once cooled to 37°C, the milk was inoculated with commercial starter culture (LYOFAST Y 259 A; SACCO, Como, Italy) alongside the prepared probiotic cultures according to Table 1. Initial *Bifidobacterium* cell densities were adjusted to 6–7 log cfu/mL prior to fermentation at 37°C until pH 4.6 was reached. Final yoghurt products were maintained at 4°C for up to 28 days. Bacteriological and physicochemical parameters were assessed weekly throughout the storage period (0, 7, 14, 21 and 28 days). Duplicate fermentations were conducted for each treatment.

Physicochemical quality evaluation

Measurement of pH

The pH values were monitored throughout fermentation and at 7-day intervals during shelf-life (0, 7, 14, 21 and 28 days) using a dairy-specific pH electrode (FC202D; HANNA Instruments Inc., USA).

Table 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*

Measurement of titratable acidity

Titratable acidity (TA) was conducted every 7 days (0, 7, 14, 21 and 28) of shelf-life at 4°C ($n = 3$). Lactic acid percentage for each sample was calculated (1):

$$\%\text{Lactic acid} = \frac{\text{titre} \times N \times 90}{M_x \times 10} \quad (1)$$

where the titre represents the volume of 0.1 N NaOH consumed during titration, N denotes the normality of the NaOH solution (0.1 N), and M_x corresponds to the original mass of yoghurt prior to dilution.

Oxidation–reduction potential analysis

Oxidation–reduction potential (ORP) was assessed using an ORP metre (HANNA® Instruments Inc., USA) with measurements performed in duplicate readings ($n = 2$) at Days 0, 7, 14, 21 and 28 of shelf-life. ORP variation magnitude was calculated using equation (2):

$$\text{ORP} = \text{ORP}_x - \text{ORP}_0 \quad (2)$$

where ORP_x denotes the ORP reading obtained on a given analysis day (Days 7, 14, 21 or 28) and ORP_0 denotes the baseline ORP reading from Day 0.

Syneresis analysis

Yoghurt samples (10 g) were dispensed into centrifuge tubes and spun for 10 min at 3500 rpm. The mass of whey obtained after separation was recorded, and the percentage of syneresis was calculated according to equation (3):

$$\%\text{Syneresis} = \frac{\text{Mass of separated whey (g)}}{\text{Initial yoghurt mass (g)}} \times 100 \quad (3)$$

Assessment of starter and probiotic bacterial viability during shelf-life

Duplicate plating ($n = 2$) of yoghurt samples was performed as specified in Table 2. The viability proportion index (VPI) for probiotic strains at the conclusion of storage (Day 28) was determined according to equation (4).

$$\text{VPI} = \frac{\text{Final cell population (cfu/g)}}{\text{Initial cell population (cfu/g)}} \quad (4)$$

Enumeration of viable bacteria using propidium monoazide-quantitative polymerase chain reaction (PMAxx-qPCR)

PMAxx-qPCR is a viability-based qPCR method in which PMAxx penetrates cells with compromised membranes, binds their DNA and prevents its amplification, thereby allowing selective quantification of DNA from only viable cells. Using the method of Marole *et al*. (2024), all species in the yoghurt, that is *Streptococcus thermophilus,*

Table 2 Culture media composition and incubation conditions for the enumeration of bacterial species from probiotic yoghurt.

Bacterial species	Media	Incubation conditions	Reference
S. thermophilus	M17 agar supplemented with 1% lactose (v/w)	37°C for 24 h	Shah (2000)
L. bulgaricus	MRS agar adjusted to pH 5.4 with 0.13% (v/v) acetic acid	37°C for 48 h, anaerobically	
Bifidobacterium spp.	MRS agar supplemented with nalidixic acid sodium salt (0.015 g/l), neomycin sulphate (0.001 g/l), lithium chloride (3 g/l), paromomycin sulphate (0.2 g/l), L-cysteine (5 g/l) (MRS-NNLP Agar)	37°C for 48 h, anaerobically	

Lactobacillus delbrueckii subsp. *bulgaricus, B. bifidum, B. breve* and *B. animalis*, were quantified individually with PMAxx-qPCR. The method was adapted: bacterial quantification was performed on Days 0 and 28, DNA extraction was performed using the ZR DNA Miniprep Kit (Zymo Research, USA), and a new standard curve was constructed for *B. animalis* using the same methodology. DNA copy numbers were calculated based on genome sizes: *B. bifidum* ATCC 29521 (2 211 767 bp), *B. breve* ATCC 15700 (2 275 660 bp) and *B. animalis* ATCC 25527 (1 932 963 bp) (Loquasto *et al*. 2011), using Avogadro's constant (6.022×10^{23}), average molecular weight of double-stranded DNA (660 Da) per base pair, and a conversion factor of 1×10^{9}. Standard curve validation for *B. animalis* confirmed the method reliability as described by Marole *et al*. (2024).

The VPI for the bacterial cultures after 28 days was calculated using equation (4).

Statistical analysis

Statistical significance of differences in both probiotic cell counts and physicochemical characteristics between yoghurt containing adapted or unadapted *Bifidobacterium* spp. was determined through ANOVA ($\alpha = 0.05$). Statistical computations were conducted using statistical Package for the Social Sciences (SPSS) Version 29 software (IBM, USA) and GraphPad Prism 10.0. Two independent experiments, each performed in duplicate, provided four replicates per treatment ($n = 4$, unless stated otherwise). A principal component analysis (PCA) was conducted using GraphPad Prism 10.0 software.

RESULTS

Fermentation characteristics of yoghurt with stress-adapted *Bifidobacterium* species

In all fermentation treatments, pH 4.6 was achieved in 3.5 h or less (Figure 1). Despite the similar endpoint, the acidification pattern differed significantly between adapted and unadapted variants ($P < 0.05$). Yoghurts containing stress-adapted variants (YBA, YRA and YAA) exhibited a more gradual and consistent decline in pH than their unadapted counterparts.

At the beginning of fermentation, *Bifidobacterium* populations across all treatments ranged between 6.0 and 7.0 log cfu/g (Figure 2). For *B. bifidum*, the unadapted variant showed a decline in viability, while the adapted variant retained significantly higher cell counts by the end of fermentation, with a 1 log cfu/g difference ($P < 0.05$).

The adapted *B. breve* increased viable cell counts during fermentation, whereas the unadapted strain declined from 6.2 to 4.6 log cfu/g ($P < 0.05$). Although not significant, both unadapted and adapted *B. animalis* increased in viable cell counts during fermentation.

Evaluation of yoghurt shelf-life with stress-adapted and unadapted *Bifidobacterium* species

Comparative physicochemical analysis of yoghurt with adapted and unadapted Bifidobacterium species

The trend in pH decline during storage remained consistent across all samples, irrespective of bacterial species or adaptation status. All yoghurt formulations had an initial pH near 4.6 on Day 0 (Figure 3). A progressive decline in pH was observed in the yoghurts over the 28-day shelf-life at 4°C period, with the most rapid decrease occurring during the first 7 days at 4°C ($P < 0.05$), where pH levels dropped to around 4.4 across all samples (Figure 3). By Day 28, the pH of all yoghurt treatments had decreased to 4.3 or lower (Figure 3).

Throughout the 28-day shelf-life at 4°C, yoghurts containing unadapted *Bifidobacterium* strains demonstrated significantly elevated lactic acid concentrations relative to adapted variants ($P < 0.05$) (Figure 3). Interestingly, yoghurts with adapted cultures consistently displayed reduced lactic acid levels at Day 14, which subsequently increased by Day 28—a trend replicated across both experimental runs. The difference remained significant on Day 28 for yoghurts containing *B. bifidum* and *B. breve* ($P < 0.05$) (Figure 3a,b). Following 28 days of shelf-life, yoghurts containing unadapted *B. bifidum* and *B. breve* (YBU and YRU) reached lactic acid levels of $1.44 \pm 0.15\%$ and $1.47 \pm 0.13\%$,

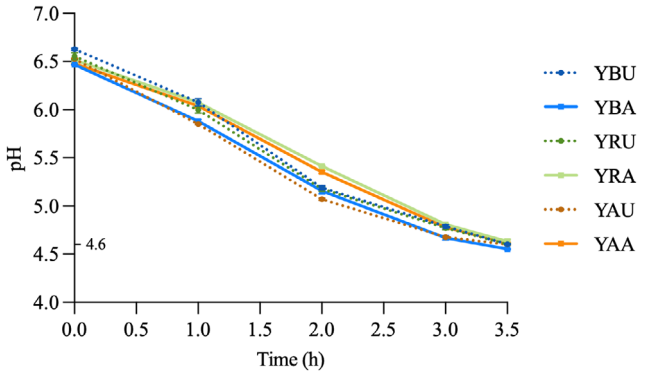

Figure 1 Decline in pH during fermentation of samples containing oxidative stress-adapted and unadapted *Bifidobacterium* species. Values are the means ± standard deviation (n = 4). YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

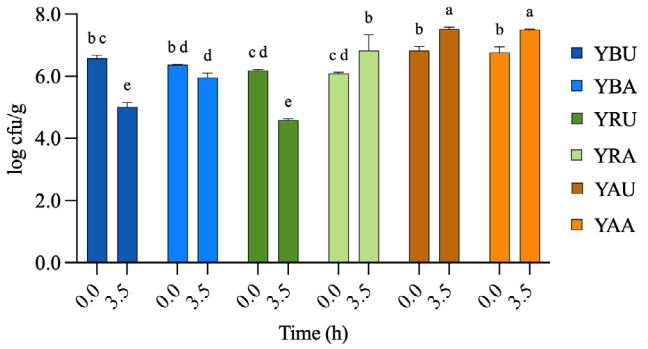

Figure 2 Viability of oxidative stress-adapted and unadapted *Bifidobacterium* spp. during fermentation, at 0.0 h (start) and 3.5 h (end of fermentation). Values are the means ± standard deviation (n = 4). Statistical analysis used two-way ANOVA (α = 0.05) comparing: (i) adapted vs unadapted variants at each time point, and (ii) start vs end of the fermentation for each treatment. Bars with different letters are significantly different ($P < 0.05$). YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

respectively, significantly exceeding those of yoghurts with adapted strains (YBA and YRA), which recorded 1.20 ± 0.11% and 1.22 ± 0.07%, respectively ($P < 0.001$) (Figure 3a,b).

ORP values increased significantly over time in all samples ($P < 0.001$), with the most marked rise occurring after Day 21 (Table 3). The rate and magnitude of ORP change differed significantly between species ($P < 0.001$), with *B. animalis* yoghurts showing the slowest increase. Notably, oxidative stress adaptation had species-specific effects, that is adapted *B. bifidum* and *B. breve* (YBA and YRA) exhibited higher ORP increases than their unadapted counterparts ($P < 0.0001$), while adapted *B. animalis* (YAA) showed significantly lower ORP changes compared with YAU ($P < 0.0001$) (Table 3).

Syneresis values for all yoghurt samples ranged from 32% to 38% at the start of shelf-life and increased over the 28 days at 4°C (data not shown). However, the syneresis of the yoghurt was not affected by the *Bifidobacterium* species nor the adaptation treatment.

Starter culture, unadapted and stress-adapted Bifidobacterium spp. viability during yoghurt shelf-life

Starter culture survival remained unaffected by either *Bifidobacterium* adaptation status or species. *S. thermophilus* populations stayed high and stable during the entire 28-day storage duration, ranging from 9.0 to 10.4 log cfu/g across all treatments (data not shown). *L. bulgaricus* persisted in all samples, albeit at reduced concentrations (3.7–4.7 log cfu/g). Notably, *L. bulgaricus* populations showed increased stability in yoghurts with adapted *B. breve*, compared with other samples, maintaining 5.8 log cfu/g post-fermentation and throughout shelf-life at 4°C (data not shown).

Figure 4 depicts viability profiles for *B. bifidum, B. breve* and *B. animalis*–unadapted and adapted variants—during 28-day refrigerated shelf-life. *B. animalis* uniquely sustained populations exceeding 6 log cfu/g during shelf-life regardless of adaptation, whereas *B. bifidum* and *B. breve* displayed progressive population reductions over time. These trends were consistent with the VPI results in Table 4.

Adaptation treatments improved post-fermentation viability for *B. bifidum* (6.0 log cfu/g) and *B. breve* (6.8 log cfu/g), compared with 5.0 log cfu/g and 4.6 log cfu/g for unadapted variants ($P < 0.0001$) (Figure 4). While both adapted and unadapted variants showed a general decline during shelf-life, the adapted variants maintained higher viability levels throughout shelf-life, as supported by VPI values showing 75.2% viability retention for adapted *B. bifidum* compared with 69.8% for unadapted variants (Table 4). Overall *B. bifidum* and *B. breve* cell counts declined during shelf-life at 4°C regardless of adaptation treatment, while *B. animalis* remained stable irrespective of adaptation.

For *B. animalis*, viable counts increased during shelf-life for both adapted and unadapted variants, reaching 7.8 and 8.9 log cfu/g by Day 28, respectively (Figure 4). The corresponding VPI values were 1.05 (adapted) and 1.8 (unadapted), indicating a 5–18% increase in viability over the 28 days of yoghurt shelf-life (Table 4).

To visualise the distinguishing features of the yoghurt samples based on microbial viability and physicochemical properties during shelf-life at 4°C a PCA was performed (Figure 5). The initial two principal components (PC1 and

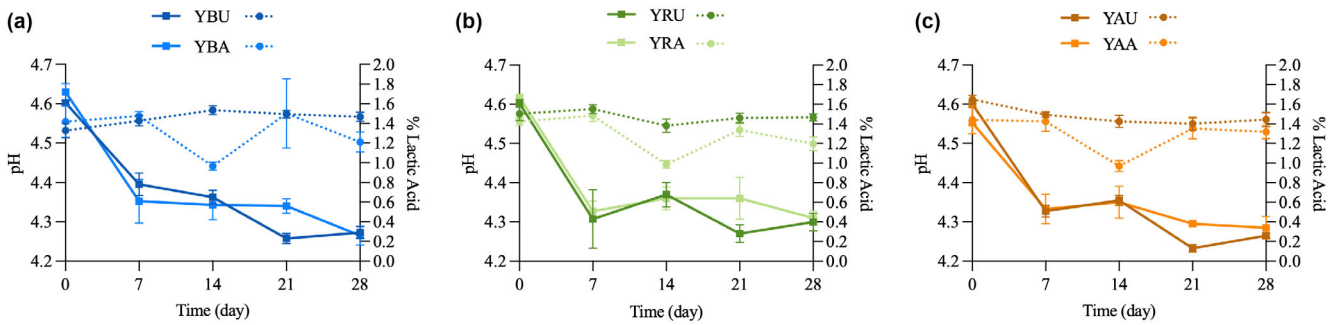

Figure 3 pH (left y-axis) and titratable acidity (% lactic acid) (right y-axis) of yoghurt samples containing oxidative stress-adapted and unadapted *Bifidobacterium* species over a 28-day shelf life at 4°C. YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*. Values are the means ± standard deviation (n = 4).

Table 3 Change in oxidation–reduction potential (ORP) (ΔmV) from Day 0 during 28-day shelf-life at 4°C of yoghurt containing unadapted or adapted *Bifidobacterium* species.

	Change in ORP (ΔmV)					
Time (day)	YBU	YBA	YRU	YRA	YAU	YAA
0–7	$128.4 \pm 23.9^{f,g}$	$140.8 \pm 21.4^{e,f}$	$154.1 \pm 5.7^{d,e}$	$79.9 \pm 5.6^{h,j}$	114.7 ± 2.8^{g}	49.9 ± 9.9^{j}
0–14	$147.0 \pm 25.6^{d,e}$	$160.7 \pm 3.8^{c,d}$	169.1 ± 6.9^{g}	110.4 ± 4.8^{g}	$130.3 \pm 1.3^{f,g}$	$80.3 \pm 4.7^{h,i}$
0–21	$169.9 \pm 25.5^{b,c}$	$171.8 \pm 19.7^{a,b}$	$194.0 \pm 6.9^{f,g}$	$120.6 \pm 2.1^{f,g}$	$154.8 \pm 1.2^{c,d}$	$97.5 \pm 6.1^{g,h}$
0–28	$152.8 \pm 26.1^{c,d,e}$	$168.8 \pm 17.7^{b,c}$	$180.1 \pm 7.1^{f,g}$	$120.2 \pm 2.2^{f,g}$	$146.8 \pm 3.2^{d,e}$	$100.6 \pm 5.8^{g,h}$

Values are the means ± standard deviation (n = 4). Values with different letters are significantly different ($P < 0.05$).
YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

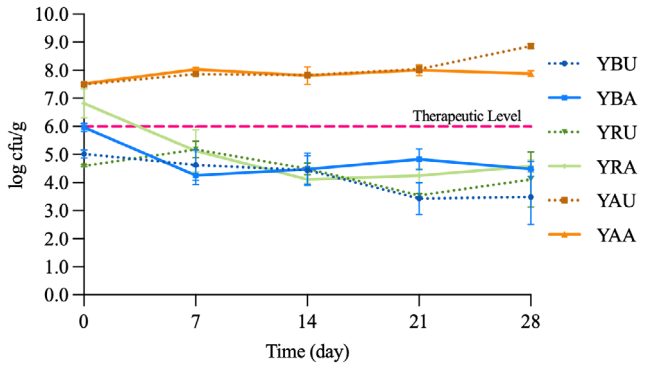

Figure 4 Viability of oxidative stress-adapted and unadapted *Bifidobacterium* spp. in yoghurt over a 28-day shelf-life at 4°C. YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*. Values are the means ± standard deviation (n = 4).

PC2) explained 59.86% of overall data variance. Yoghurt samples at Day 0 clearly distinguished from the remainder of the shelf-life at 4°C, with progressive pH reduction during the shelf-life being the key differentiating characteristic. Moreover, PCA demonstrated positive associations between ORP and shelf-life time, while both variables showed inverse relationships with the pH. Despite these trends, no clear differentiation emerged between yoghurts formulated with stress-adapted and unadapted *Bifidobacterium* species. However, both *B. animalis* samples (YAA and YAU) retained high *Bifidobacterium* counts even as TA rose.

PMAxx-qPCR as an alternative to standard plate counting for bacterial quantification in mixed-species yoghurt

PMAxx-qPCR-based viability counts of *S. thermophilus* were between 10.0 and 11.0 log cfu/g across all treatments, regardless of *Bifidobacterium* species or adaptation status (Figure 6a). The PMAxx-qPCR-based *L. bulgaricus* viability counts differed between Day 0 and Day 28 across treatments (Figure 6b). In yoghurt samples YBA, YBU and YRU, *L. bulgaricus* counts declined from Day 0 to Day 28. Yoghurt sample YRA containing adapted *B. breve* was an exception, showing higher *L. bulgaricus* counts at Day 28 (7.8 log cfu/g) compared with Day 0. Notably, lower counts

Table 4 Effect of stress adaptation on the viability proportion index (VPI) of *Bifidobacterium* spp. in yoghurt at the end of shelf-life at 4°C (Day 28), as measured by MRS-NNLP and PMAxx-qPCR, respectively.

	$VPI_{(MRS\text{-}NNLP)}$		$VPI_{(PMAxx\text{-}qPCR)}$	
Species	Unadapted	Stress-adapted	Unadapted	Stress-adapted
B. bifidum	0.698 ± 0.211	0.752 ± 0.050	0.808 ± 0.004	0.806 ± 0.006
B. breve	0.895 ± 0.213	0.675 ± 0.062	0.971 ± 0.015	0.947 ± 0.002
B. animalis	1.182 ± 0.019	1.047 ± 0.012	0.900 ± 0.037	0.940 ± 0.007

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

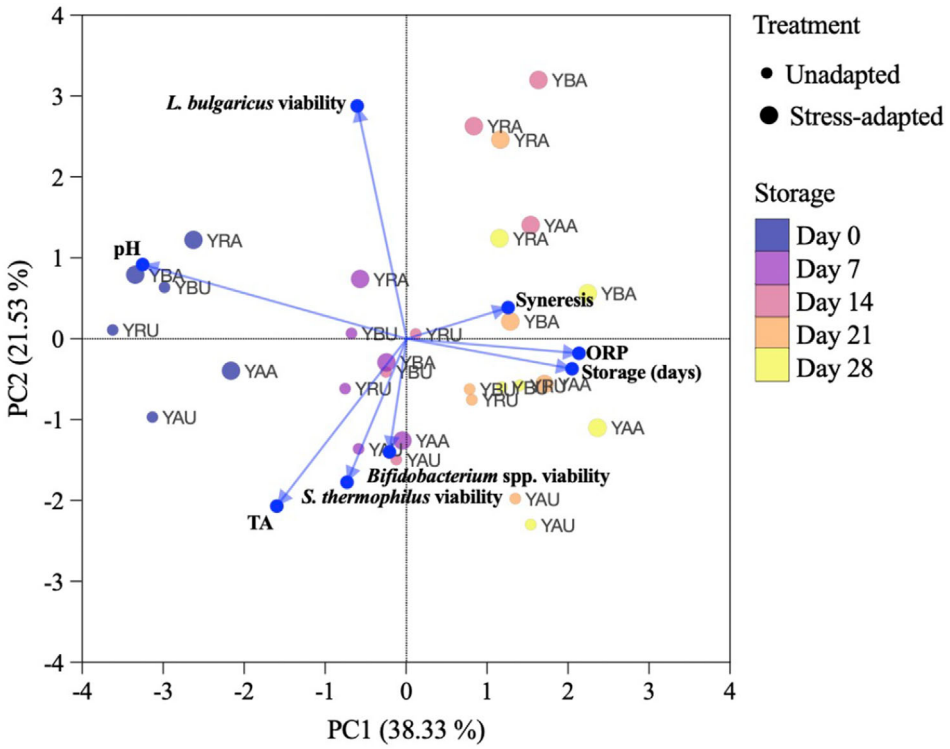

Figure 5 Principal component analysis (PCA) of physicochemical characteristics [pH, titratable acidity (TA), oxidation–reduction potential (ORP)] and viability of yoghurt bacteria (*S. thermophilus* and *L. bulgaricus*) and unadapted or stress- adapted *Bifidobacterium* spp. in yoghurt during shelf-life at 4°C (Days 0, 7, 14, 21 and 28). Percentage of variance of PC1 and PC2 are indicated in parentheses. Arrows indicate the contribution of each variable to PC1 and PC2. YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

were observed after 28 days for yoghurt samples containing *B. animalis* in both adapted and unadapted treatments (Figure 6b).

The PMAxx-qPCR-based *Bifidobacterium* viability counts were generally lower at Day 28 than at Day 0. The most differences were observed for *B. bifidum*, both unadapted and adapted, and to a lesser extent *B. animalis* (Figure 7). This is supported by the VPI indices showing a 20% decline in *B. bifidum* viability for both variants—unadapted and adapted—while *B. breve* showed a marginal decline of 3–5% (Table 4). Comparison of PMAxx-qPCR counts between adapted and unadapted variants for all *Bifidobacterium* spp. showed no differences at both Day 0 and Day 28 (Figure 7). Throughout the shelf-life, the three *Bifidobacterium* spp. maintained the therapeutic minimum probiotic level in yoghurt (Figure 7).

Enumeration of bacterial populations using the PMAxx-qPCR method showed a strong correlation with the SPC method (Pearson $r = 0.9621$, $P < 0.0001$) (Figure 8a). However, PMAxx-qPCR consistently yielded higher viability

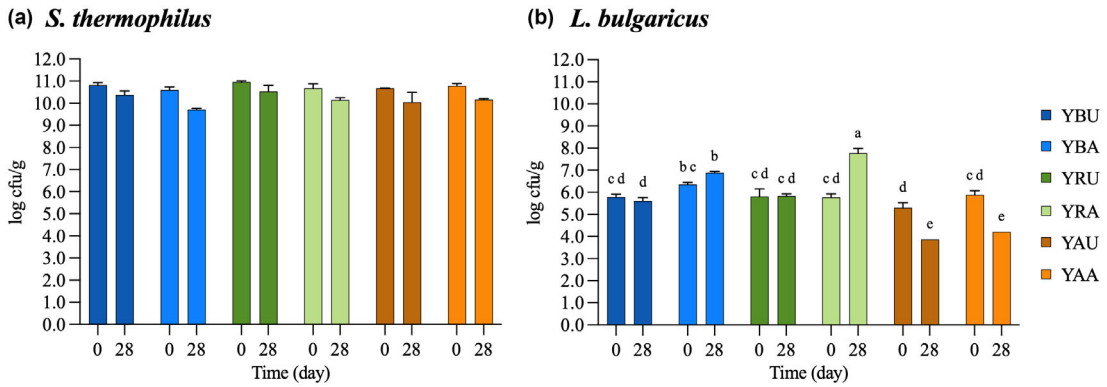

Figure 6 Viability of *S. thermophilus* (a) and *L. bulgaricus* (b) in yoghurt prepared with unadapted or stress-adapted *B. bifidum, B. breve* or *B. animalis*, on Days 0 and 28 of shelf-life at 4°C, as determined by PMAxx-qPCR. Values are the means ± standard deviation (n = 2). YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

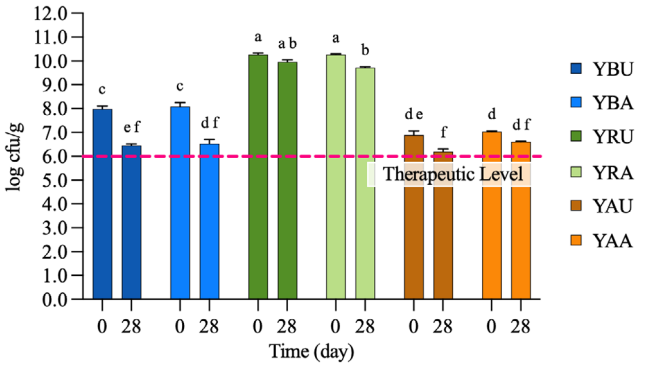

Figure 7 Probiotic viability in yoghurt prepared with unadapted or stress-adapted *B. bifidum*, *B. breve* or *B. animalis*, on Days 0 and 28 of shelf-life at 4°C, as determined by PMAxx-qPCR. Values are the means ± standard deviation (n = 2). YAA, yoghurt with stress-adapted *B. animalis*; YAU, yoghurt with unadapted *B. animalis*; YBA, yoghurt with stress-adapted *B. bifidum*; YBU, yoghurt with unadapted *B. bifidum*; YRA, yoghurt with stress-adapted *B. breve*; YRU, yoghurt with unadapted *B. breve*.

counts, with a mean difference of 27% compared with SPC (Figure 8b).

Estimated shelf-life based on enumeration methods

Shelf-life estimates derived from both SPC and PMAxx-qPCR are presented in Table 5. These estimates incorporated *Bifidobacterium* spp. counts obtained on Days 0, 7, 14, 21 and 28 at 4°C of shelf-life (Figures 2 and 7), evaluated against the FAO/WHO (2003) recommended therapeutic threshold of 6 log cfu/g.

Standard plate counts revealed that unadapted *B. bifidum* and *B. breve* reached 5 log cfu/g immediately post-fermentation, while their adapted counterparts achieved 6.0 and 6.8 log cfu/g, respectively (Figure 2). After 1 week of storage, adapted *B. bifidum* and *B. breve* populations decreased to 4.3 and 5.1 log cfu/g, respectively (Table 5 and Figure 2). In contrast, *B. animalis* maintained concentrations above 6 log cfu/g throughout the storage period, regardless of adaptation treatment (Table 5).

PMAxx-qPCR enumeration revealed *Bifidobacterium* spp. populations maintained above 6 log cfu/g at the beginning and Day 28 of shelf-life, regardless of species identity or adaptation status (Figure 7 and Table 5).

DISCUSSION

Yoghurt fermentation characteristics affected by stress-adapted *Bifidobacterium* spp.

The results demonstrate significant inter-species variation in coping with increasing acid stress during fermentation. The accelerated acidification rate apparently surpassed the adaptive capacity of unadapted *B. bifidum* and *B. breve*, resulting in considerable viability reductions by the completion of fermentation, similar to findings by El-Dieb *et al.* (2012), that documented *B. bifidum* population declines during yoghurt fermentation linked to rapid pH reduction. Although acid tolerance mechanisms in *Bifidobacterium* spp. are well documented (Wei *et al.* 2019; Schöpping *et al.* 2022), maintaining viability during active fermentation remains a challenge. Furthermore, species-specific responses to oxidative stress adaptation revealed distinct patterns. *B. bifidum* demonstrated reduced ability to tolerate acidity regardless of adaptation treatment, showing a decline during fermentation in both unadapted and adapted variants, underscoring its inherent acid sensitivity.

In contrast, *B. breve* showed distinctly different behaviour, with the adapted variant demonstrating enhanced survival and even growth during fermentation while the unadapted variant declined. The stress adaptation treatment may have

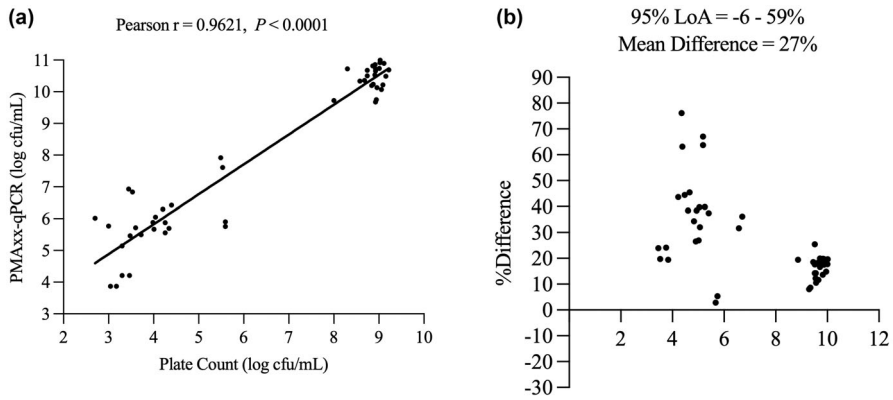

Figure 8 (a) Simple linear regression and (b) Bland–Altman method of comparison (% Difference vs. Average) of the PMAxx-qPCR method compared with the standard plate count method (n = 48). The Bland–Altman comparison = expressed as a percentage relative difference: [100 × ((PMAxx-qPCR count − Plate count))/(average)].

Table 5 Effect of stress adaptation on the predicted shelf-life[a] at 4°C of probiotic yoghurt containing *B. bifidum, B. breve* or *B. animalis,* based on the recommended minimum viable level for probiotics in yoghurt.

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

[a]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 2003). This criterion applies to microorganisms 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.

resulted in constitutively active stress response pathways that diverted cellular resources from rapid acid production towards survival mechanisms, creating more controlled fermentation conditions. This cross-protection mechanism appears particularly effective in *B. breve*, enabling better tolerance to multiple stressors during yoghurt fermentation (Maus and Ingham 2003; Jin *et al*. 2015). Adapted *B. bifidum* maintained higher viable counts, while adapted *B. breve* showed increased cell numbers during fermentation—suggesting an improved metabolic and physiological state during yoghurt fermentation.

B. animalis showed increased viability during fermentation, regardless of adaptation treatment, suggesting a robust intrinsic tolerance to acidic conditions. This reflects characteristic species traits, including natural acidic environment resilience or rapid environmental adaptation capabilities, representing valuable attributes for probiotic applications (Ruiz *et al*. 2011).

Influence of stress-adapted *Bifidobacterium* spp. on probiotic yoghurt shelf-life and quality

Yoghurts with unadapted *Bifidobacterium* spp. exhibited higher TA compared with those containing adapted *Bifidobacterium* species. This finding differs from previous findings that associated greater acidification with acid-adapted *Bifidobacterium* spp. (Oğuz *et al*. 2023). The adaptation treatment employed in this study potentially facilitated the development of mechanisms governing acid production more efficiently, preventing excessive acidification that could compromise long-term survival (Schöpping *et al*. 2022). Adapted variants potentially modulate lactic acid production more effectively, avoiding over-acidification and preserving a more stable yoghurt-matrix, which may enhance consumer acceptance (Settachaimongkon *et al*. 2015). *L. bulgaricus* is typically the primary contributor to post-fermentation acidification during shelf-life. The observed differences in lactic acid production between unadapted and adapted variants may reflect how the different *Bifidobacterium* spp. and their adaptation status influence *L. bulgaricus* metabolic activity through species interactions, nutrient competition or metabolic cross-feeding, thereby affecting overall acidification patterns during the 28-day shelf-life (Donkor *et al*. 2006; Ulmer *et al*. 2022).

Despite continued pH decline, the consistent decrease in TA on Day 14 in all adapted samples suggests differential metabolic activity between adapted and unadapted strains. This apparent contradiction between pH and TA trends indicates that factors beyond lactic acid production may influence acidification during the shelf-life period, warranting further investigation.

ORP serves as a critical indicator of the oxidative environment in yoghurt, with elevated values indicating

conditions that promote oxidative stress and potentially compromise probiotic viability. Although ORP does not directly measure dissolved oxygen, it reflects the redox balance of yoghurt (Bulat and Topcu 2019). During the shelf-life, oxygen dissolves from the headspace into the yoghurt. In the absence of sufficient antioxidants or reducing agents to neutralise the dissolved oxygen, ORP values are expected to increase progressively. High ORP may promote reactive oxygen species (ROS) generation, imposing oxidative stress on *Bifidobacterium* species in yoghurt. The ORP increased progressively during shelf-life across all treatments, likely due to ongoing microbial metabolism and oxygen incorporation (Martin-Dejardin *et al*. 2013). Adapted *B. animalis* formulations exhibited the most gradual ORP elevation during shelf-life, potentially attributable to reduced metabolic rates and inherent oxidative stress resistance (Oberg *et al*. 2011; Schöpping *et al*. 2022). While limited specific information is available for *B*. animalis, *B. animalis* subsp. *lactis*, which is closely related, possesses oxygen-sequestering mechanisms, which may similarly contribute to the stable redox conditions observed in this study (Ruiz *et al*. 2012). Adaptation may have further strengthened these defences, improving redox stability throughout shelf-life. Consequently, yoghurt containing adapted *B. animalis* exhibited more stable ORP levels throughout shelf-life than the unadapted strain. However, the limited change in ORP between adapted and unadapted samples for other species suggests a species-specific effect of the adaptation treatment.

Streptococcus thermophilus levels remained elevated throughout shelf-life across all yoghurt formulations, reflecting its known industrial robustness (Settachaimongkon *et al*. 2015; Yerlikaya *et al*. 2021). Notably, adapted *B. breve* appeared to promote *L. bulgaricus* viability during storage, suggesting a potential synergistic interaction not previously reported. This may be attributed to stress response mechanisms that indirectly benefited *L. bulgaricus*. Comparable findings were reported by Yerlikaya *et al*. (2021), demonstrating enhanced *L. bulgaricus* viability in probiotic yoghurt during shelf-life. Another explanation could be metabolic cross-feeding, such as increased availability of branched-chain amino acid (BCAA). Adaptation may have upregulated BCAA synthesis in *B. breve* (Ulmer *et al*. 2022), enriching the yoghurt with amino acids that support *L. bulgaricus* growth. Further investigation using transcriptomic or metabolic approaches would be valuable to elucidate this interaction.

Bifidobacterium animalis maintained consistent viability throughout refrigerated storage in both adapted and control treatments, highlighting its natural robustness against oxidative stress and other adverse conditions within yoghurt. This observation is consistent with Lamoureux *et al*. (2002), who similarly documented persistent *B. animalis* viability during shelf-life, with counts consistently exceeding 6.0 log cfu/g.

Extensive investigations by Oberg *et al*. (2013) examining the genetic foundations of constitutive and inducible oxidative stress responses in *Bifidobacterium* spp. established that stress response induction exhibits inherent species- and strain-level specificity. This species-specific variation explains why *B. animalis* demonstrates superior survival compared with *B. bifidum* and *B. breve* in the current study. The superior survival of *B. animalis* can be attributed to several specific physiological and genetic mechanisms that are intrinsic to this species.

Oberg *et al*. (2013) demonstrated that *B. animalis* subsp. *lactis* possesses highly efficient oxidative stress defence systems, particularly involving thioredoxin reductase and peroxiredoxin, key mechanisms for detoxifying hydrogen peroxide and other ROS encountered during yoghurt fermentation and shelf-life. Additionally, this species exhibits efficient protein stabilisation and turnover systems that are critical for maintaining cellular integrity under oxidative stress conditions (Oberg *et al*. 2013).

Furthermore, strain-specific genetic variations contribute to the observed stress tolerance. Oberg *et al*. (2013) identified that differences in H_2O_2 resistance among *Bifidobacterium* strains may result from mutations affecting long-chain fatty acid coenzyme A (CoA) ligase genes, influencing membrane composition and cellular stress responses. Wei *et al*. (2019) corroborated this, demonstrating that acid stress triggers membrane fatty acid profile alterations, with compositional shifts towards specific fatty acids enhancing overall stress tolerance. Beyond oxidative stress resistance, the acid tolerance mechanisms of *B. animalis* are equally important for its survival in the acidic yoghurt environment (pH $\sim$ 4.3–4.6).

Conversely, the declining *B. bifidum* and *B. breve* populations observed within the first week of shelf-life at 4°C may be attributed to continued acidification following fermentation and significant ORP variations. Furthermore, metabolic by-products from yoghurt starter cultures—such as lactic acid, H_2O_2, bacteriocins and volatile compounds—could have compromised the survival of these species (Mortazavian *et al*. 2011). Oğuz *et al*. (2023) similarly documented declining probiotic viability with increasing acidity. These observations emphasise *B. bifidum* and *B. breve* susceptibility to post-fermentation acid stress and starter culture interactions, underscoring the requirement for strategies minimising acidification while preserving probiotic viability throughout yoghurt shelf-life (Schöpping *et al*. 2022).

Consequently, the higher viable counts of adapted *B. bifidum* and *B. breve* relative to unadapted strains throughout shelf-life indicate successful adaptation-mediated viability enhancement. Multiple investigators have demonstrated *Bifidobacterium* spp. adaptation to stress factors, particularly oxidative stress, yielding variants tolerating higher stress intensities (Mozzetti *et al*. 2010; Oberg and Broadbent 2016). These findings suggest that the adaptation process strengthened stress defence mechanisms while simultaneously providing cross-protective effects against

additional stressors, such as low pH, thereby enhancing the overall robustness of *B. bifidum* and *B. breve* (Maus and Ingham 2003; Wei *et al*. 2019). The improved stress response capacity in adapted *B. bifidum* and *B. breve* translated to better maintenance of culturable cell populations.

Alternatively, the marginal improvement in plate counts may point to ephemeral adaptation effects that mainly enhance stress tolerance during fermentation. Maus and Ingham (2003) hypothesised that conditions during yoghurt fermentation could reverse the stress response enhancements obtained during stress adaptation. That said, adaptive responses to stress in *Bifidobacterium* show marked species- and stressor-specific differences (Maus and Ingham 2003; Saarela *et al*. 2004; Mozzetti *et al*. 2010; Schöpping *et al*. 2022).

The PCA analysis reveals that stress adaptation treatment influences yoghurt physicochemical and microbiological attributes in a highly species-specific manner. This emphasises the intricate, strain-dependent response patterns and adaptation pathways exhibited by probiotics facing oxidative and other stresses within the yoghurt environment (Settachaimongkon *et al*. 2015). Importantly, the adaptation treatment demonstrated benefits for *B. bifidum* and *B. breve*, with adapted strains maintaining higher viability levels than their unadapted counterparts, particularly evident during fermentation and extending through Day 21 of shelf-life at 4°C. This represents a significant finding with practical applications for the dairy industry, as it shows that oxidative stress adaptation can enhance the technological performance of these challenging probiotic species.

Furthermore, PCA analysis supports the strong tolerance of *B. animalis* to oxidative and additional stressors found in yoghurt. Oberg *et al*. (2013) observed significant stress responses and tolerance in *B. animalis* subsp. *lactis* DSM 10140, which is closely related phylogenetically to the *B. animalis* strain used in this study, particularly in relation to H_2O_2 stress. The minimal observable adaptation treatment effects suggest the protocol may have been insufficient to enhance permanent oxidative stress tolerance, with possible reversion to baseline states post-adaptation. These observations highlight the need for further investigation into species-specific stress adaptation strategies for *Bifidobacterium* species.

While *B. animalis* is widely used, different *Bifidobacterium* species offer distinct probiotic benefits. *B. bifidum* and *B. breve* have unique metabolic capabilities and strain-specific health effects that cannot be replicated by *B. animalis* alone. For instance, *B. bifidum* is particularly associated with infant gut health and immune development, while *B. breve* shows specific benefits for certain digestive conditions (Nyanzi *et al*. 2021; Sibanda *et al*. 2024).

These findings highlight the potential for implementing stress adaptation strategies in industrial probiotic production to enhance the technological performance of traditionally challenging *Bifidobacterium* species in fermented dairy applications.

PMAxx-qPCR enhances detection of viable probiotic bacteria in yoghurt

This study represents application of the PMAxx-qPCR method for quantifying viable probiotics in mixed-species yoghurt, following the initial development and validation by Marole *et al*. (2024). The method demonstrated excellent performance characteristics, with a strong correlation between PMAxx-qPCR and SPC methods, confirming its efficacy (Figure S1). Additionally, our study extends their validation by applying the method to effectively quantify *S. thermophilus, L. bulgaricus* and *Bifidobacterium* spp. in yoghurt samples. The strong correlation between SPCs and PMAxx-qPCR results offers a more comprehensive probiotic viability assessment in yoghurt.

PMAxx-qPCR enumeration revealed all three *Bifidobacterium* species consistently maintained populations exceeding the recommended probiotic minimum [6 log cfu/g (FAO/WHO 2003)] throughout the complete shelf-life duration, contrasting with viable counts suggesting substantial reductions.

These differences suggest the presence of a viable but nonculturable (VBNC) population, especially within *B. bifidum* and *B. breve* (Lahtinen *et al*. 2008; Dias *et al*. 2020; Marole *et al*. 2024). We propose that the extent of VBNC *Bifidobacterium* populations at the start (Day 0) and end (Day 28) of shelf-life can be determined by analysing the percentage relative mean variation between conventional plating and PMAxx-qPCR quantification through Bland–Altman analysis (Gagnon *et al*. 2015). According to these calculations, roughly 27% of *Bifidobacterium* cells were in the VBNC state. Although the VBNC phenomenon has been documented in *Bifidobacterium* during food processing (Amor *et al*. 2002; Lahtinen *et al*. 2008), the current findings demonstrate its significance in commercial fermented dairy applications.

Species-specific responses to oxidative stress were evident. *B. breve* maintained elevated viability independent of adaptation status, likely transitioning to a VBNC state as a survival mechanism against oxidative and additional stressors during shelf-life (Lahtinen *et al*. 2006, 2008). This supports its potential as a robust probiotic candidate. In contrast, *B. bifidum* declined markedly in both PMAxx-qPCR and SPCs, confirming its sensitivity to oxidative stress. *B. animalis* showed stable counts across both methods, suggesting it did not enter a VBNC state and reinforcing its known resilience (Oberg *et al*. 2013), supporting its suitability for probiotic-enriched yoghurt.

These findings highlight the limitations of relying solely on plate count methods to evaluate probiotic survival in fermented dairy foods. Traditionally, a loss of culturability has been assumed to indicate cell death. However, it may

signify a transition to the VBNC state, serving as a survival strategy that could help maintain the viability and functionality of probiotics. Furthermore, the ability of VBNC *Bifidobacterium* cells to reverse their unculturable state has not been thoroughly studied. This area needs further research to ensure the therapeutic efficacy of probiotics incorporated into yoghurt, ultimately benefiting consumer health.

Subjecting *Bifidobacterium* spp. to stress adaptation significantly enhanced survival during yoghurt manufacturing and shelf-life, as shown by the shelf-life analysis. According to the recommended therapeutic threshold of 6 log cfu/g (FAO/WHO 2003), both unadapted *B. bifidum* and *B. breve* had a shelf-life of 0 days, meaning they fell below the therapeutic level immediately after fermentation. In contrast, their adapted counterparts achieved shelf-lives extending between 0 and 7 days, representing a significant technological improvement for these traditionally challenging species. These improvements likely reflect specific cellular adaptations, such as upregulation of stress response genes, enhanced carbohydrate metabolism or membrane modifications associated with oxidative stress adaptation (Oberg *et al*. 2013; Schöpping *et al*. 2022). Both unadapted and adapted *B. animalis* sustained populations exceeding 6 log cfu/g throughout the shelf-life, confirming its superior probiotic suitability in yoghurt and emphasising species-specific stress tolerance characteristics.

Nevertheless, PMAxx-qPCR analysis demonstrated that all *Bifidobacterium* species maintained therapeutic concentrations throughout 28-day shelf-life when VBNC populations were included, fundamentally altering shelf-life evaluation. This finding demonstrates that traditional plate count methods underestimate probiotic viability and may lead to premature product rejection. The PMAxx-qPCR technique's ability to detect both culturable and VBNC populations offers a more comprehensive and accurate assessment of probiotic functionality. This capability enhances shelf-life estimations and allows for more precise labelling of probiotic content in fermented dairy products. Since probiotics are defined by their ongoing health benefits to the host (FAO/WHO 2003), future research should focus on whether VBNC *Bifidobacterium* species retain their functionality in the gastrointestinal tract.

CONCLUSION

This study shows that *Bifidobacterium* species adapted to oxidative stress, resulting in moderate improvements in probiotic viability during yoghurt fermentation and the early phase of refrigerated shelf-life at 4°C, particularly for *B. breve* and *B. bifidum*. Adapted strains exhibited modified acidification patterns during fermentation, with slower pH decline and reduced lactic acid production during shelf-life compared with unadapted counterparts. *B. animalis* exhibited superior stability throughout, regardless of adaptation, highlighting species-specific responses and resilience. The application of PMAxx-qPCR revealed higher bacterial counts than standard plate methods, indicating the method's sensitivity, as well as the potential formation of VBNCs. These findings support the potential of stress adaptation strategies and advanced viability assays for developing probiotic yoghurts with improved shelf-life and compliance with minimum recommended therapeutic levels. Further research should investigate the molecular mechanisms underlying these species-specific adaptation responses, including gene expression profiling of stress response markers, membrane composition changes and antioxidant enzyme activities, as well as evaluate VBNC cell functionality. Additional work is also needed to explore strain-specific or multi-stressor adaptation approaches for long-term viability enhancement in dairy applications. These insights provide a foundation for future innovation in the design of robust, effective and consumer-aligned probiotic yoghurt products.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Elna Maria Buys: Conceptualization; funding acquisition; writing – review and editing; visualization; methodology; project administration; supervision; resources; validation. **Ursula Louise Thomashoff:** Investigation; formal analysis; data curation; writing – original draft; methodology; validation; writing – review and editing. **Thulani Sibanda:** Conceptualization; methodology; validation; visualization; supervision; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

The following supporting information is available for this article:

Figure S1 (a) Melt curves showing primer specificity and relative amplicon quantities of serially diluted (10^0–10^7) *Bifidobacterium* spp. DNA and the resultant standard curve. (b) 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* ATCC25527. Each point represents the mean ± standard deviation of CT values ($n = 2$).

