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ORIGINAL PAPER

Intrinsic and inducible resistance to hydrogen peroxide in *Bifidobacterium* species

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Abstract Interest in, and use of, bifidobacteria as a probiotic delivered in functional foods has increased dramatically in recent years. As a result of their anaerobic nature, oxidative stress can pose a major challenge to maintaining viability of bifidobacteria during functional food storage. To better understand the oxidative stress response in two industrially important bifidobacteria species, we examined the response of three strains of *B. longum* and three strains of *B. animalis* subsp. *lactis* to hydrogen peroxide (H_2O_2). Each strain was exposed to a range of H_2O_2 concentrations (0–10 mM) to evaluate and compare intrinsic resistance to H_2O_2. Next, strains were tested for the presence of an inducible oxidative stress response by exposure to a sublethal H_2O_2 concentration for 20 or 60 min followed by challenge at a lethal H_2O_2 concentration. Results showed *B. longum* subsp. *infantis* ATCC 15697 had the highest level of intrinsic H_2O_2 resistance of all strains tested and *B. animalis* subsp. *lactis* BL-04 had the highest resistance among *B. lactis* strains. Inducible H_2O_2 resistance was detected in four strains, *B. longum* NCC2705, *B. longum* D2957, *B. lactis* RH-1, and *B. lactis* BL-04. Other strains showed either no difference or increased sensitivity to H_2O_2 after induction treatments. These data indicate that intrinsic and inducible resistance to hydrogen peroxide is strain specific in *B. longum* and *B. lactis* and suggest that for some strains, sublethal H_2O_2 treatments might help increase cell resistance to oxidative damage during production and storage of probiotic-containing foods.

Keywords *Bifidobacterium* · Hydrogen peroxide · Stress response

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Introduction

Foods and food ingredients with "bioactive" properties, which are defined by their ability to impact human health in a manner not based solely on their nutritional value, have increased in popularity among consumers in the last decade. One example involves "probiotic" bacteria, which are "living organisms that, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition" [12]. Currently, species of *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added to commercial bioactive products [33]. Many species of bifidobacteria are used as probiotics, but two of the most important commercial species are *B. longum* and *B. animalis* subsp. *lactis* (henceforth described as *B. lactis*).

Although bifidobacteria are relatively minor components of the normal gastrointestinal (GI) microbiota in human adults, research indicates that some strains can promote or provide several health-related functions, including host resistance to infectious microbes, anti-carcinogenic activities, and improved nutritional efficiency [2, 41]. Moreover, certain species of bifidobacteria are

major components of the GI microbiota in healthy, breast-fed infants, and recent work suggests that the composition of GI microbiota in infants and children may influence the development of diarrheal, inflammatory, and allergic diseases [31].

No conclusive data are available on the minimal effective dose of probiotics in humans, but results from several clinical trials suggest a direct dose–effect correlation [23, 26, 35]. As a result, the current World Health Organization (WHO) definition of probiotics emphasizes the need for administration of the probiotics in "adequate amounts" [27]. Thus, successful application of probiotic bifidobacteria in foods is not only dependent upon the functionality of the strain, but also on the development of technologies to ensure their survival in high numbers during food processing and maintaining those high numbers during storage.

Challenges associated with probiotic delivery are exacerbated by the fact that these cells are commonly exposed to unfavorable environmental conditions during the manufacture or storage of most food-based delivery systems for bifidobacteria [29]. For example, efforts to secure and maintain high numbers of viable bifidobacteria in bioactive food products are commonly impeded by the intrinsic and extrinsic properties of the food such as dehydration (low a_w), high or low temperature, low pH, high sodium chloride levels, or presence of oxygen, all of which may be deleterious to bacteria [7, 9, 29, 39]. To address this problem, processors may employ very large inocula or add specific growth promoters or protectants [25, 29, 44]. Additionally, the ability of bacteria to resist environmental extremes is generally affected by growth phase, with stationary-phase cells showing far greater resistance than mid-log-phase cells [45]. As a result, industrial production of probiotic cultures is typically performed in large fermenters under rigid pH and temperature control, and cells are harvested at late-log or early stationary-phase growth to maximize cell biomass and vigor [22, 30].

Oxygen toxicity results from cell exposure to activated oxygen compounds such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals, which induce peptide breaks, oxidation of sulfhydryl groups in proteins, and oxidation of membrane lipids [6]. Although H_2O_2 is the most stable of these molecules, dissociation and interaction with cellular components can form organic peroxides, which can initiate a chain reaction of oxidation [13]. Most bifidobacteria lack genes for catalase and superoxide dismutase, which are used by many bacteria to detoxify H_2O_2 and superoxide, respectively. Nonetheless, *Bifidobacterium* species have been shown to produce inhibitory levels of H_2O_2 when incubated in the presence of oxygen [10]. Moreover, bifidobacteria are commonly added to yogurt products and the starter cultures used to manufacture yogurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, have also been shown to produce inhibitory concentrations of H_2O_2 when grown under conditions such as those encountered during bioactive food production and storage [40].

Several studies have shown that environmental stress resistance in many microorganisms, including some bifidobacteria, may be dramatically improved by deliberate induction of an adaptive or inducible stress response [4, 5, 8, 11, 15, 22, 30, 37, 42]. These inducible stress responses are characterized by the transient induction of genes that encode general and specific stress proteins (e.g., chaperones and ATP-dependent proteases) and corresponding regulatory proteins [3, 16, 21, 32, 34, 46, 47].

The knowledge that sublethal stress treatments can promote cell robustness is already exploited in the manufacture and use of probiotic cultures [39], but a more detailed understanding of environmental adaptation by bifidobacteria to oxidative stress would likely reveal new strategies to improve the industrial stability, performance, and utility of these probiotics. Thus, the purpose of this research was to investigate the intrinsic and inducible H_2O_2 stress resistance in several industrially important strains of *B. longum* and *B. lactis*.

Materials and methods

Bacterial strains and culture conditions

Strains of *B. longum* and *B. lactis* selected for use in this study are listed in Table 1. Strains were maintained as glycerol freezer stocks at −80°C, and working cultures were prepared by two successive transfers (1% inoculum, v/v) into peptonized milk medium (MP5) (3% proteose peptone, 1.4% glucose, 1.7% yeast extract, 0.1% Tween 80, 0.45% sodium chloride, 0.05% cysteine HCl) with anaerobic incubation at 37°C for 18 h.

Table 1 Bifidobacteria selected for this study

Species and strain	Description [reference]
B. animalis subsp. *lactis*	
BL-04	Industrial probiotic strain [3]
DSM 10140	Industrial probiotic and type strain [3]
RH-1	Industrial probiotic strain
B. longum	
NCC2705	Industrial probiotic strain; isolated from human infant [36]
D2957	Industrial probiotic strain
B. longum subsp. *infantis*	
ATCC 15697	Industrial probiotic strain; isolated from human infant [38]

Batch cultures of each strain were prepared for H_2O_2 resistance studies by a 1% (v/v) inoculation of working cultures, diluted to an OD_{600} of 1.0 in MP5 broth, into 1 L of MP5 in a 1-L New Brunswick BioFlo III fermenter (New Brunswick Scientific, Edison, New Jersey), with an agitation rate of 100 rpm and an incubation temperature of 37°C. A gas mixture of 5% carbon dioxide and 95% nitrogen was continuously passed over the headspace of the fermenter to achieve anaerobic conditions, and the pH was maintained at 6.5 by automatic addition of 15% (v/v) ammonium hydroxide. The cultures were incubated until the cells reached early stationary phase (approximately 12 h for the *B. lactis* strains and 14 h for the *B. longum* strains).

Intrinsic hydrogen peroxide resistance

Intrinsic H_2O_2 resistance of each culture was measured in 10 mL MP5 medium with addition of H_2O_2 at concentrations of 0.65, 1.3, 2.25, 5.25, and 10.5 mM, plus a control which contained no H_2O_2. Early stationary-phase cells were grown in batch culture as described, then collected by centrifugation at 3,500×*g* and diluted 1:100 to obtain a cell concentration of 10^4–10^6 colony forming units (CFU)/mL. The cells were inoculated at 1% (v/v) into MP5 with different H_2O_2 concentrations and placed in an anaerobic jar (BD, Franklin Lakes, NJ). The jar headspace was flushed with a mixture of 5% carbon dioxide/95% nitrogen then placed at 37°C. Aliquots (1 mL) were collected every hour for 6 h starting at time 0, serially diluted in sterile 0.1% peptone, and plated on MRS (Difco, Sparks, MD) agar plates supplemented with 0.05% filter sterilized cysteine (MRS + C) using the spread plate technique. Agar plates were incubated in anaerobic jars at 37°C for 48 h before enumeration. Replicates were performed in quadruplicate. After each sampling time, the tubes were placed back in an anaerobic jar and the headspace was flushed with gas as described. The H_2O_2 concentration of each MP5 tube was assayed at time 0 using the SensoLyte ADHP hydrogen peroxide colorimetric assay kit (AnaSpec, San Jose, CA) following the manufacturer's protocol. All H_2O_2 assays were performed in quadruplicate using optical grade removable strip 96-well plates (Thermo Scientific, Vantaa, Finland) and absorbance at 576 nm was read on a Spectramax Plus 384 plate reader (Molecular Devices Corp., Sunnyvale, CA). To standardize the results, the measured peroxide concentration before inoculation and the CFU/mL after 1 h (for each strain) were fitted into a least-squares linear regression model with the 95% confidence interval of the slopes used to determine significant differences between the strains [14].

For inducible stress response testing, lethal stress treatments for each strain were defined as the minimum H_2O_2 concentration at which there were no recoverable cells over the 6-h incubation period. Sublethal stress treatments for each strain were defined as the highest H_2O_2 concentration that resulted in no more than a 1 $\log_{10}$ decrease in cell numbers during the 6-h exposure [30].

Screening for inducible H_2O_2 stress resistance

Cells were grown to early stationary phase in batch culture, then 10-mL samples were collected and centrifuged at 3,500×*g* for 5 min. The cell pellet was suspended in 10 mL MP5 broth warmed to 37°C that contained a sublethal H_2O_2 concentration (1.25 mM for all strains) and incubated for 20 or 60 min at 37°C in an anaerobic jar flushed with a 5% carbon dioxide/95% nitrogen gas mixture. After the 20- or 60-min sublethal H_2O_2 exposure, the cells were collected by centrifugation, and suspended in 10 mL of MP5 warmed to 37°C that contained either 2.55 mM or 5.25 mM H_2O_2 as the lethal challenge and incubated anaerobically at 37°C. Samples (1 mL) were taken after 0, 15, and 30 min of exposure, and plated as described for intrinsic resistance studies. Controls were prepared the same way as test cultures, except that no H_2O_2 was added to the MP5 medium used for the 20- or 60-min incubations prior to lethal H_2O_2 exposure. Replicates were performed in quadruplicate.

Any difference in a strain's ability to withstand a particular lethal stress treatment after a sublethal H_2O_2 exposure was expressed as a percent survival, which was calculated by dividing the $\log_{10}$ CFU/mL of surviving cells after a 30-min lethal H_2O_2 challenge by the $\log_{10}$ CFU/mL of cells after a 0-min lethal H_2O_2 exposure. To determine if the calculated percent survival was significant, means from the induced strain were compared to control means using a one-tailed two-sample *t* test without pooled variance with $\alpha = 0.05$ [14].

Results

As shown in Fig. 1, all strains of bifidobacteria tested were killed within 2- to 3-h incubation in MP5 with 5.25 mM added H_2O_2, but heterogeneity in cell survival was observed at lower H_2O_2 concentrations. The $\log_{10}$ CFU data collected over the 6-h incubation (Fig. 1) was used to select sublethal (1.25 mM) and lethal H_2O_2 (2.55 and 5.25 mM) concentrations for oxidative stress induction and H_2O_2 challenge, respectively, for all six strains.

Because H_2O_2 is a strong oxidizing agent, we anticipated there would be a loss in concentration upon addition to broth medium and preliminary tests confirmed the concentration of H_2O_2 decreased from 20 to 60% between samples (data not included). This finding indicated that

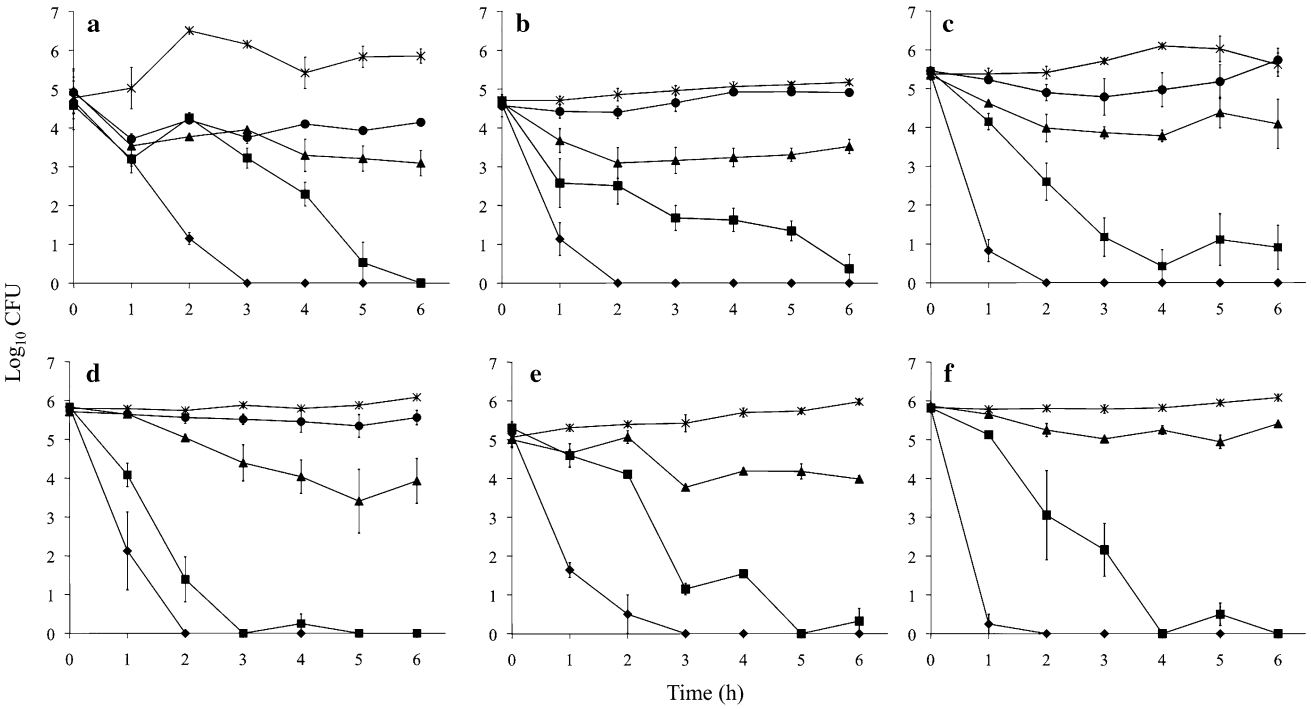

Fig. 1 Survival of bifidobacteria over 6 h in MP5 broth medium with different concentrations of added H_2O_2. *X axis*, time (h). *Y axis*, $\log_{10}$ CFU/mL. **a** *B. infantis* ATCC 15697; **b** *B. longum* NCC2705; **c** *B. longum* D2957; **d** *B. lactis* BL-04; **e** *B. lactis* RH-1; **f** *B. lactis* DSM 10140. *Crosses* control; *circles* 0.66 mM H_2O_2; *triangles* 1.25 mM H_2O_2; *squares* 2.25 mM H_2O_2; *diamonds* 5.25 mM H_2O_2. *Error bars* correspond to the standard error of the mean (SEM)

direct measurement of H_2O_2 in the medium, before cell exposure, was important for accurate comparisons between strains. To facilitate such comparisons, the measured H_2O_2 concentrations prior to inoculation and the $\log_{10}$ CFU/mL after 1-h exposure (because cell survival data beyond 1 h were nonlinear at some H_2O_2 concentrations; Fig. 1) were fitted into a linear model in which the calculated slope of the plotted line corresponds to H_2O_2 resistance (similar to *Z* value determination in thermal destruction of an organism [24]), and a steeper slope shows faster cell death at increasing concentrations of H_2O_2 (Fig. 2). On the basis of the linear model (Fig. 2), *B. longum* subsp. *infantis* ATCC 15697 had significantly higher ($P < 0.05$) intrinsic H_2O_2 resistance than all the strains tested. *B. lactis* BL-04, *B. longum* NCC2705, and *B. lactis* RH-1 showed an intermediate level of intrinsic H_2O_2 resistance, whereas *B. longum* D2957 and *B. lactis* DSM 10140 displayed the lowest intrinsic resistance to H_2O_2 (Fig. 2).

Experiments to screen *B. longum* and *B. lactis* strains for inducible H_2O_2 stress resistance revealed that most strains displayed a decreased percent survival after the lethal challenge compared to control cells (Figs. 3 and 4). These results show that some strains were unable to mount an inducible stress response under the conditions tested and, in some cases, cells that were exposed to sublethal H_2O_2 were more sensitive to lethal H_2O_2 concentrations than control cells. However, 60-min sublethal H_2O_2 treatment with *B. longum* NCC2705 significantly ($P < 0.05$) increased the survival of this strain at both lethal H_2O_2 concentrations tested (Fig. 4), and a significant ($P < 0.05$) increase in survival was also recorded for *B. longum* NCC2705 and *B. longum* D2957 given a 20-min treatment followed by a lethal challenge at 2.55 mM H_2O_2 (Fig. 3a). Among the *B. lactis* strains, *B. lactis* RH-1 and *B. lactis* BL-04 cells given a 20-min induction treatment showed a significant ($P < 0.05$) increase in survival after 5.25 mM H_2O_2 challenge (Fig. 4b).

Discussion

Results from this study indicate that intrinsic and inducible H_2O_2 resistance is both species and strain specific in *B. longum* and *B. lactis*. Previous studies have investigated H_2O_2 resistance in bifidobacteria and lactic acid bacteria (LAB) at a wide range of H_2O_2 concentrations under static conditions in differing buffer solutions [17–20, 28, 43]. However, our study design sought to explore H_2O_2 resistance of each strain in a milk peptone-based growth medium similar to those used for commercial production of probiotic cultures instead of buffer, so that cells had an opportunity for active metabolism during exposure.

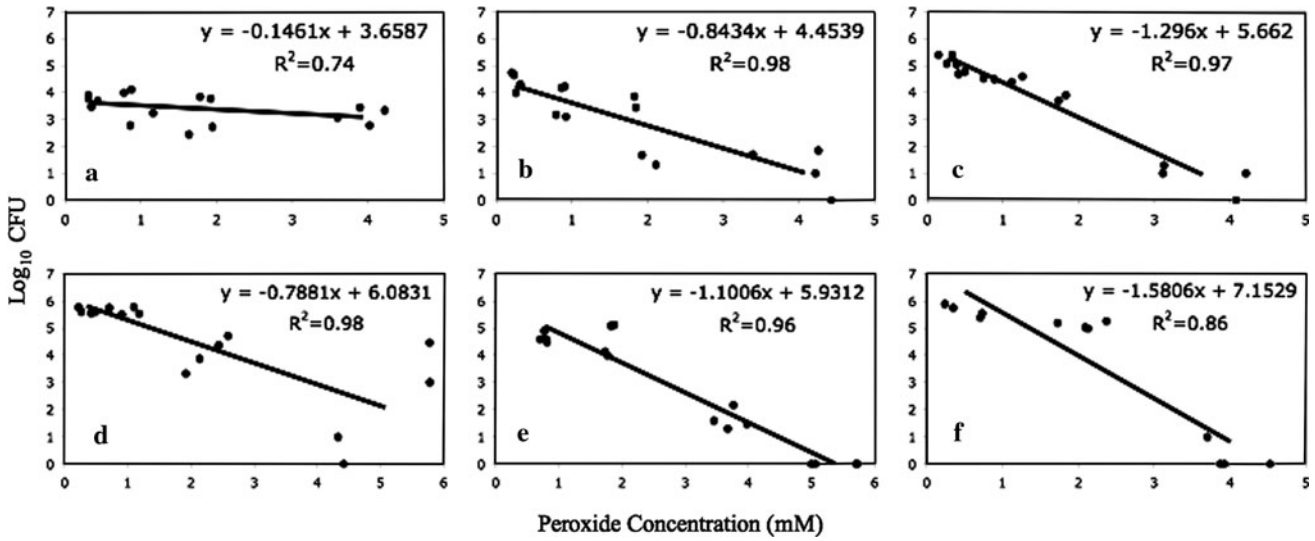

Fig. 2 Linear regression plots of intrinsic H_2O_2 resistance. *X axis*, measured H_2O_2 concentration in MP5 medium before inoculation. *Y axis*, $\log_{10}$ CFU/mL after 1-h incubation. **a** *B. infantis* ATCC 15697; **b** *B. longum* NCC2705; **c** *B. longum* D2957; **d** *B. lactis* BL-04; **e** *B. lactis* RH-1; **f** *B. lactis* DSM 10140

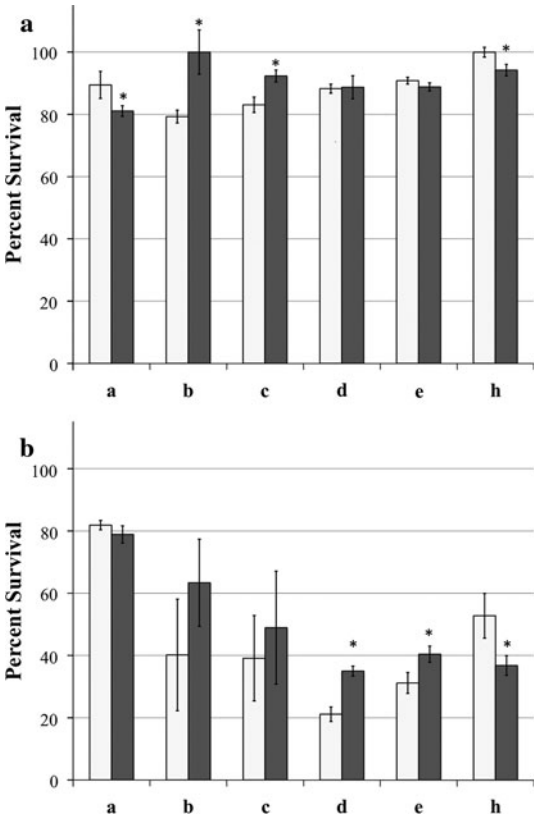

Fig. 3 Percent survival of bifidobacteria after a 20-min experimental stress response induction at 1.25 mM H_2O_2 challenged at lethal concentrations of **a** 2.55 mM H_2O_2 and **b** 5.25 mM H_2O_2. *Lanes a*, *B. infantis* ATCC 15697; *b*, *B. longum* NCC2705; *c*, *B. longum* D2957; *d*, *B. lactis* BL-04; *e*, *B. lactis* RH-1; *f*, *B. lactis* DSM 10140. *Open square*s, control; *filled squares*, induced. Each value is the mean of four replicates. *Error bars* correspond to the standard error of the mean (SEM). *Asterisks* denote bars that have a mean percent survival significantly different ($P < 0.05$) from control

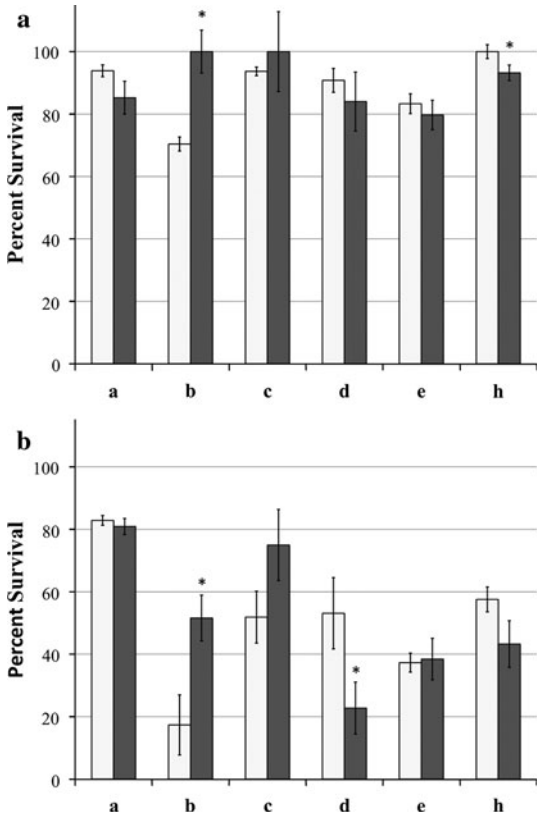

Fig. 4 Percent survival of bifidobacteria after 60-min experimental stress response induction at 1.25 mM H_2O_2 challenged at lethal concentrations of **a** 2.55 mM H_2O_2 and **b** 5.25 mM H_2O_2. *Lanes a*, *B. infantis* ATCC 15697; *b*, *B. longum* NCC2705; *c*, *B. longum* D2957; *d*, *B. lactis* BL-04; *e*, *B. lactis* RH-1; *f*, *B. lactis* DSM 10140. *Open squares*, control; *filled squares*, induced. Each value is the mean of four replicates. *Error bars* correspond to the standard error of the mean (SEM). *Asterisks* denote bars that have a mean percent survival significantly different ($P < 0.05$) from control

Comparison of the whole genome sequences for *B. lactis* DSM 10140, *B. lactis* BL-04, *B. longum* NCC2705, and *B. infantis* ATCC 15697 showed they each lacked genes for the classical oxidative stress response enzymes superoxide dismutase, catalase, and a true peroxidase [3, 38]. Further genetic analysis of these strains reveals that they contain genes for flavin proteins, although they lack a gene for flavin reductase, as well as genes for a thioredoxin reductase/thioredoxin system and a peroxiredoxin. The thioredoxin reductase systems works in conjunction with NADPH to maintain the redox potential in the cell for proper disulfide bond formation in proteins, and serves as an electron donor for enzymes such as ribonucleotide reductase [1]. This system also donates electrons to peroxiredoxin for the reduction of H_2O_2–H_2O. Interestingly, comparison of the whole genome sequence of *B. lactis* BL-04 and *B. lactis* DSM 10140 shows that they are almost identical [3]. Our data show that there are large differences between the intrinsic H_2O_2 resistance and inducible H_2O_2 stress responses of these strains, which should be attributable to one or more of their minor genetic differences. Further research is underway to explore this observation.

Although certain strains showed higher H_2O_2 resistance than others, the lethal H_2O_2 concentration for all strains was relatively low (2.55–5.25 mM). These values are within the concentration range of H_2O_2 produced by lactic starter cultures during the manufacture of yogurt and other bioactive foods [40], which underscores the need for technologies to enhance H_2O_2 resistance in bifidobacteria. Our results suggest that a sublethal H_2O_2 exposure could be used to enhance H_2O_2 resistance of some strains (e.g., *B. longum* NCC2705 and *B. lactis* BL-04), and increase their survival in functional foods. Additionally, more detailed studies of inducible H_2O_2 stress resistance in these strains may reveal strategies to enhance H_2O_2 resistance in a broader range of strains.

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