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Transcriptome analysis of *Bifidobacterium longum* strains that show a differential response to hydrogen peroxide stress

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ABSTRACT

Consumer and commercial interest in foods containing probiotic bifidobacteria is increasing. However, because bifidobacteria are anaerobic, oxidative stress can diminish cell viability during production and storage of bioactive foods. We previously found *Bifidobacterium longum* strain NCC2705 had significantly greater intrinsic and inducible resistance to hydrogen peroxide (H_2O_2) than strain D2957. Here, we explored the basis for these differences by examining the transcriptional responses of both strains to sub-lethal H_2O_2 exposure for 5- or 60-min. Strain NCC2705 had 288 genes that were differentially expressed after the 5-min treatment and 114 differentially expressed genes after the 60-min treatment. In contrast, strain D2957 had only 21 and 90 differentially expressed genes after the 5- and 60-min treatments, respectively. Both strains showed up-regulation of genes coding enzymes implicated in oxidative stress resistance, such as thioredoxin, thioredoxin reductase, peroxiredoxin, ferredoxin, glutaredoxin, and anaerobic ribonucleotide reductase, but induction levels were typically highest in NCC2705. Compared to D2957, NCC2705 also had more up-regulated genes involved in transcriptional regulation and more down-regulated genes involved in sugar transport and metabolism. These results provide a greater understanding of the molecular basis for oxidative stress resistance in *B. longum* and the factors that contribute to strain-to-strain variability in survival in bioactive food products.

1. Introduction

Consumption of food or food ingredients with bioactive properties has increased in recent years and probiotic bacteria represent one of the most promising categories of bioactive food ingredients. The term probiotic refers to living microorganisms which, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition (Fontana et al., 2013). Currently, bacteria from the genera *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added to functional foods. Bifidobacteria are Gram-positive, non-acid-fast, non-spore forming, non-motile, anaerobic, catalase negative rods of irregular shape, with a G+C content of 55–67%, and are part of the normal gastrointestinal flora in human adults (Klaassens et al., 2009; Vaughan et al., 2005). Bifidobacteria are thought to promote or provide several health related functions, including a decrease in severity of the side effects associated with antibiotics use, a reduced incidence of infection in patients receiving irradiation therapy, a decrease in the duration of diarrhea due to various etiologies, improved lactose digestion, a reduced frequency of allergic reactions, normalization of blood lipid composition, and a decrease in gut transit time (Chen et al., 2010; Dong et al., 2010; Zhang et al., 2009; Stanton et al., 2001; Bermudez-Brito et al., 2012). Although no conclusive data is available on a minimal effective dose of probiotics in humans, results from several clinical trials suggest a direct dose-effect correlation (Waller et al., 2011; Meance et al., 2001; Reid et al., 2001). In practice this means that bifidobacteria need to be delivered at very high concentrations in bioactive foods to function as a probiotic. At present, yogurt or fermented milks are the most common vehicle foods for delivery of probiotic bifidobacteria, but cheese, ice cream, infant formula, fruit juice, and other foods are also used to a lesser extent (Makinen et al., 2012). One of the major hurdles to production and storage of bioactive foods containing bifidobacteria as a probiotic is oxidative stress. This is because bifidobacteria are anaerobic and lack common enzymes such as superoxide dismutase that detoxify oxidative free radicals in the presence of oxygen (Vries and Stouthamer, 1969; Barrangou et al., 2009; Ruiz et al., 2011; Oberg et al., 2011). We previously found *Bifidobacterium longum* strain NCC2705 had greater intrinsic and inducible resistance to H_2O_2 than strain D2957. Specifically, 60 min

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Table 1
Target genes oligonucleotide primers for RT-PCR.

Protein function (Gene ID)	Primer sequence (5′–3′)		Amplicon size (bp)	Annealing temp (°C)
	Forward	Reverse		
Peroxiredoxin (BL0615)	GTGGCTGAGCGTGGCGACTT	ACCTGGTCGCCGTGCTCGTA	146	61.5
Glutaredoxin (BL0668)	CACCAAGCGCCAGCTCACCA	GCGTGATGACCACCGGAGCC	122	61.5
Thioredoxin (RBLN00690) (BLD_0988)	GCGCGTTCGGCCCGATTTTC	GGGCCAGATCCTGGTTGGCG	99	61.5
Ferredoxin (BL01563)	TACGAGGGTTCCCGCTCGCT	AAGATGGCCTCGGTGGGGCA	89	61.5
Ferridoxin (BL1725)	GGCTACGCGGGTGCATTGGT	CCGGGGTGAAACGTGGGTCG	105	61.5
Anaerobic Ribonuclease reductase (BL1752)	TCAAGGGGCGTTACACCGGC	GGCGCGAGCCACATCGTACA	97	61.5
DnaK (BL0520)	CCCAGCGTCAGGCCACCAAG	GCTGCGGTCGGCTCGTTGAT	79	61.5

exposure to sublethal concentration (1.25 mM) of H_2O_2 was shown to significantly improve survival ($P < 0.05$) of *B. longum* NCC2705 at lethal (5.25 mM) H_2O_2 concentrations, but a similar response was not detected with strain D2957 (Oberg et al., 2011). Additionally, *B. longum* NCC2705 showed a 1.5-fold higher intrinsic H_2O_2 resistance than D2957 (Oberg et al., 2011). The purpose of this study was to explore the physiological basis for these differences by determining the transcriptional responses of *B. longum* NCC2705 and D2957 to sublethal H_2O_2 exposure.

2. Materials and methods

2.1. Media, strains and culture conditions

Bacterial strains were maintained as glycerol freezer stocks at −80 °C, and working cultures were prepared by two successive transfers (1% innoculum, vol/vol) into peptonized milk medium (MP5; Oberg et al., 2011) with anaerobic incubation at 37 °C for 18 h. Batch cultures of each strain were prepared by dilution of the working culture to an absorbance at 600 nm (A_{600}) of 1.0 in MP5 medium, then inoculated at 1% (vol/vol) into 1 L of MP5 in a New Brunswick BioFlo III fermenter (New Brunswik Scientific, Edison, New Jersey). Cells were incubated at 37 °C with an agitation rate of 100 rpm to prevent sedimentation. A gas mixture of 5% CO_2 and 95% N_2 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% (vol/vol) NH_4OH. Bifidobacteria were incubated until the cells reached early stationary-phase (approximately 12 h) (Oberg et al., 2011).

2.2. RNA isolation

RNA isolation was performed as previously described (Oberg et al., 2013). Cells were grown in batch culture under pH control to early stationary phase and then samples (5 mL) were harvested by centrifugation at 7500 × *g* for 10 min. The cell pellets were suspended in 50 mL of pre-warmed MP5 media containing a sublethal H_2O_2 concentration of 1.25 mM (16) for 5 (T1) or 60 (T2) min. Immediately after treatment, 100 mL of RNAprotect bacterial reagent (Qiagen, Inc., Valencia, CA) was added to the cell suspensions to stop transcription and prevent mRNA degradation. A control sample was also prepared which was not exposed to H_2O_2. Cells in RNA protect were held at room temperature for 10 min, then collected by centrifugation at 9500 × *g* for 10 min and stored at −20 °C until RNA isolation.

Cell pellets were thawed at room temperature and suspended in 900 μL of a lysis solution containing 20 mg lysozyme (Sigma–Aldrich) and 20 U of mutanolysin (Sigma–Aldrich) per mL in 1 mM TE buffer adjusted to pH 7.6. Samples were incubated for 30 min at 37 °C on a shaker incubator at 240 rpm, after which 20 μL of proteinase K (Omega Bio-Tek Inc., Norcross, GA) (>600 mAU/ml) was added and the samples were returned to the shaker/incubator for 30 min. The RNA was then isolated using the Aurum total RNA mini kit (Biorad, Hurcules, CA) following the vendor's recommended procedures. The quantity of recovered RNA was measured with a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA) and the quality of the RNA was assayed using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Samples that had sufficient quantities (>10 μg) of quality RNA were stored at −80 °C until needed.

2.3. Synthesis and labeling of cDNA

cDNA synthesis and labeling was performed as described previously (Oberg et al., 2013). cDNA was synthesized and labeled as recommended by the Affymetrix (Santa Clara, CA) protocol for prokaryotic target preparation in the GeneChip Expression Analysis Technical Manual (media.affymetrix.com). The cDNA was fragmented into approximately 50–100 bp using DNAse I and labeled with GeneChip DNA labeling reagent (Affymetrix, Santa Clara, CA) and terminal deoxynucleotidyl transferase (Promega, Madison, WI). Fragmentation labeling efficiency was measured by gel shift assay.

2.4. DNA microarrays hybridization and statistical analysis

DNA microarray hybridization and statistical analysis was performed as described previously (Oberg et al., 2013). Sample hybridization was performed at the Center for Integrated Biosystems at Utah State University against a custom Affymetrix bifidobacterial DNA microarray designed to include 3113 shared and unique chromosomal genes predicted to occur in *B. longum* NCC2705 and D2957. The only predicted coding sequences not included in the microarray design were redundant transposases and rRNA genes. Hybridization was performed according to the Affymetrix protocol for prokaryotic target hybridization in the GeneChip expression analysis technical manual using a hybridization temperature of 50 °C. The DNA microarrays were scanned using the HP GeneArray scanner (Affymetrix, Santa Clara, CA) to generate raw intensity values for each probe.

Statistical analysis of microarray data was performed using Bioconductor (www.bioconductor.org) in the open source statistical platform R (www.r-project.org). The raw probe data was preprocessed using the RMA-MS method (Stevens et al., 2008) and filtered to only include genes that had a high signal intensity and a low coefficient of variation. To test for differential expression, the preprocessed, filtered data was analyzed using the limma/eBayes method (Scholtens and von Heydebreck, 2005). Genes were determined to be significantly differentially expressed if they had a false discovery rate corrected *P*-value <0.05. The significantly differentially expressed genes were grouped according to function and by treatment times and strain.

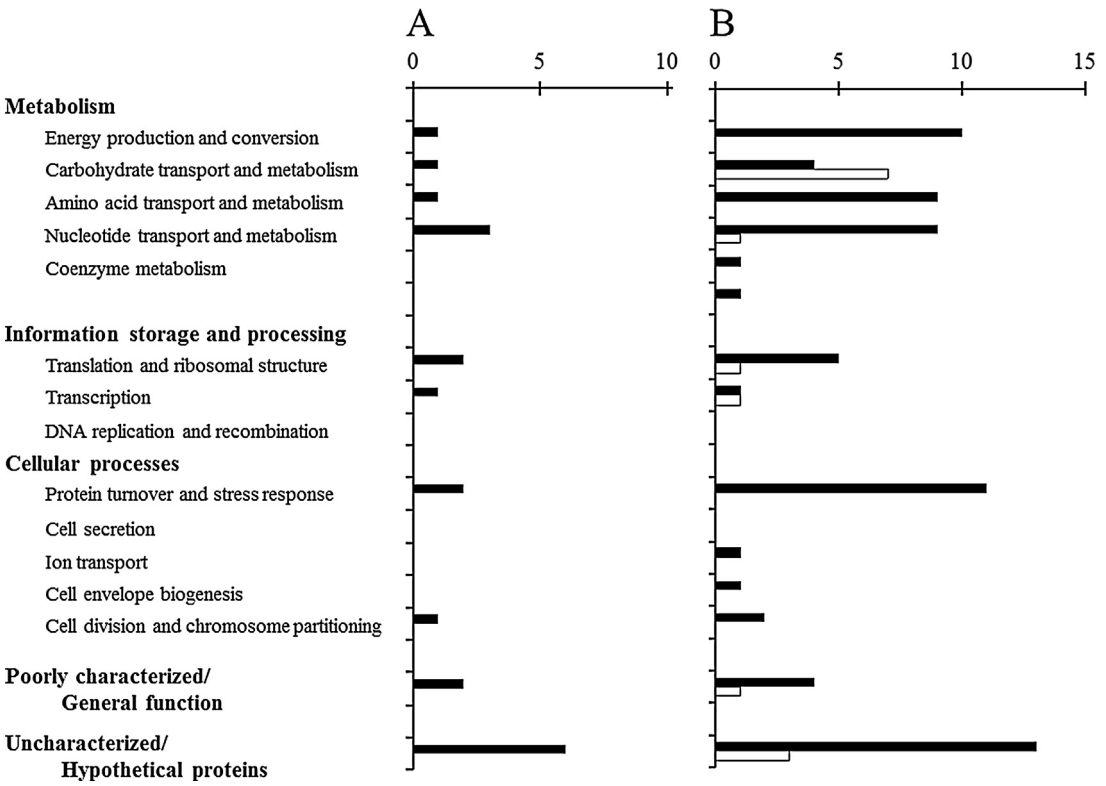

Fig. 1. Numbers of *B. longum* D2957 genes, grouped according to functional category, that were significantly upregulated (black bars) or downregulated (white bars) after 1.25 mM H_2O_2 exposure for 5 min (panel A) or 60 min (panel B).

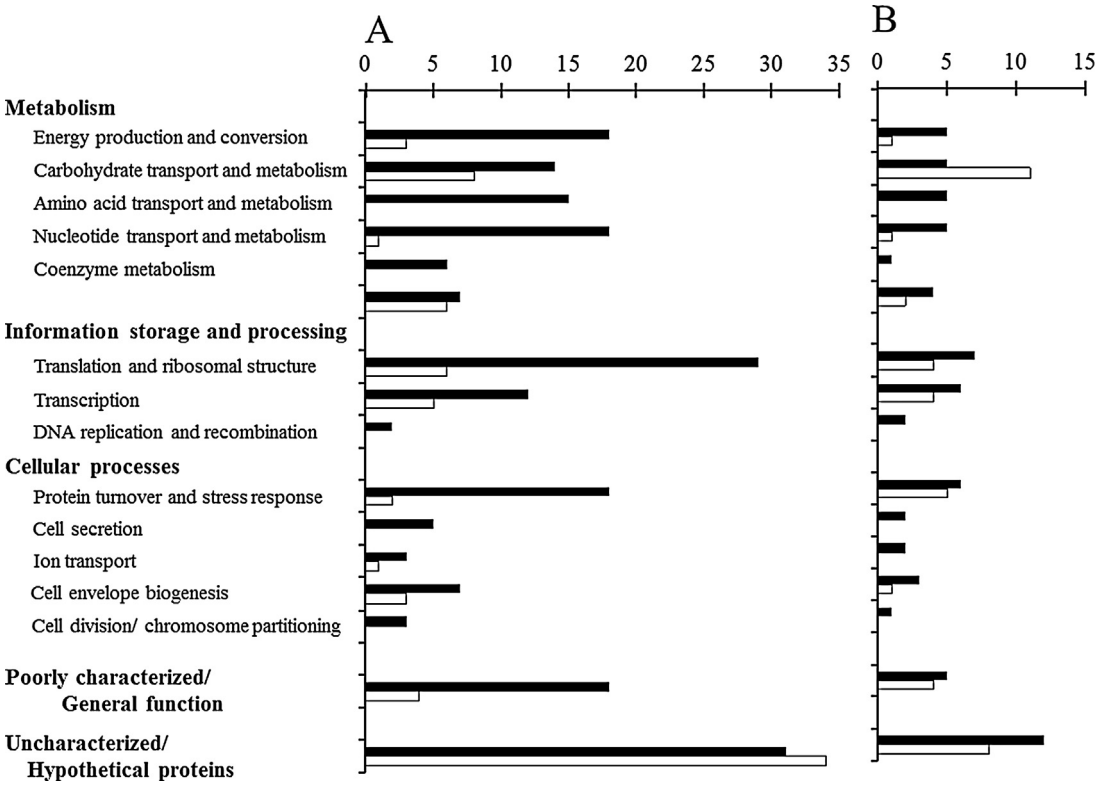

Fig 2. Numbers of *B. longum* NCC2705 genes, grouped according to functional category, that were significantly upregulated (black bars) or downregulated (white bars) after 1.25 mM H_2O_2 exposure for 5 min (panel A) or 60 min (panel B).

Table 2
Differentially expressed genes associated with oxidative stress response of *B. longum* strains NCC2705 and D2957. Results are expressed as a $\log_2$ fold change after 5-min (T1) and 60-min (T2) compared to control (untreated) cells.

Gene ID	Predicted function	NCC2705		D2957	
		T1	T2	T1	T2
BL0555	Trypsin-like serine proteases, typically periplasmic	1.68	n.s.	n.s.	n.s.
BL0781	Putative intracellular protease/amidase	0.94	0.96	n.s.	n.s.
BL0944	ATP-dependent endopeptidase clp proteolytic subunit clpP	n.s.	n.s.	n.s.	0.96
BL0945	Protease subunit of ATP-dependent Clp proteases	0.79	n.s.	n.s.	1.48
BL1682	ATP-dependent Zn proteases	0.99	n.s.	n.s.	n.s.
BL0139	NAD(P)H-dependent FMN reductase	n.s.	n.s.	n.s.	1.92
BL0399	Protoporphyrinogen oxidase	1.06	n.s.	n.s.	n.s.
BL0552	NADPH-dependent glutamate synthase beta chain and related oxidoreductases	n.s.	1.03	n.s.	n.s.
BL0614	Thioredoxin reductase	1.92	1.56	n.s.	0.88
BL0615	Peroxiredoxin	1.36	1.29	n.s.	1.16
BL0668	Glutaredoxin and related proteins	1.79	1.59	1.57	1.49
BL0669	NrdI protein/ribonucleotide reductase stimulatory protein	1.42	n.s.	n.s.	n.s.
BL0670	Ribonucleotide reductase, alpha subunit	0.92	n.s.	n.s.	n.s.
BL0671	Ribonucleoside-diphosphate reductase beta chain	1.40	n.s.	n.s.	0.73
BL1563	Ferredoxin	0.86	n.s.	n.s.	n.s.
BL1750	Exodeoxyribonuclease VII small subunit	0.75	1.10	n.s.	n.s.
BL1752	Oxygen-sensitive ribonucleoside-triphosphate reductase	1.88	1.59	n.s.	n.s.
BL1753	Anaerobic ribonucleoside-triphosphate reductase activating protein	0.95	0.89	n.s.	n.s.
BLD_0988	Thioredoxin	n.s.	n.s.	n.s.	1.52
BL0001	Cold shock proteins	0.75	n.s.	n.s.	0.85
BL0002	60 kDa chaperonin GROEL	n.s.	n.s.	n.s.	1.09
BL0010	ATPases with chaperone activity, ATP-binding subunit	0.89	n.s.	n.s.	n.s.
BL0355	Predicted nuclease of the RecB family	−0.82	n.s.	n.s.	n.s.
BL0517	Molecular chaperone (small heat shock protein)	n.s.	0.92	n.s.	n.s.
BL0519	ATPases with chaperone activity, ATP-binding subunit	n.s.	−1.08	n.s.	n.s.
BL0520	DnaJ-class molecular chaperone	n.s.	−1.28	n.s.	n.s.
BL1250	Molecular chaperone	n.s.	−1.72	n.s.	n.s.
BL1558	10 kDa chaperonin GROES	n.s.	n.s.	n.s.	0.98
BLD_0001	Cold shock protein	n.s.	n.s.	1.51	1.06
BL1664	Universal stress protein UspA and related nucleotide-binding proteins	−1.15	−1.74	n.s.	n.s.
BLD_1771	Stress-responsive transcriptional regulator PspC	0.84	n.s.	n.s.	n.s.

n.s.: Not significant.

2.5. Microarray validation by RT-PCR

To validate the microarray data, quantitative real-time PCR (RT-PRC) was performed as described by Smeianov et al. (2007) for 6 different genes (Table 1) using cDNA produced after each treatment. A log-fold change (LFC) was calculated between control and treatment samples, and graphed vs. the LFC calculated from the microarray data.

2.6. Microarray data accession number

Microarray hybridization data have been deposited in the Gene Expression Omnibus under accession number GSE44709.

2.7. Whole genome sequencing of B. longum D2957

Total genomic DNA was isolated from strain D2957 using the Masterpure Gram Positive DNA purification kit (Epicenter Biotechnologies, Madison WI) then high-throughput whole-genome shotgun DNA sequencing and assembly was performed at the Utah State University Center for Integrated Biosystems using the Roche 454 GS Titanium pyrosequencer platform. The sequence data was assembled into a 2.33 Mbp draft genome consisting of 13 contigs, and automated genome annotation was performed using the RAST algorithm (rast.nmpdr.org).

The whole genome shotgun sequence for *B. longum* D2957 has been deposited at DDBJ/EMBL/GenBank under the accession AQGL00000000. The version described in this paper is the first version, AQGL01000000.

2.8. Membrane fatty acid analysis

To determine whether H_2O_2 exposure altered cytoplasmic membrane fatty acid (CMFA) composition, cells were grown in batch culture as described, and treated with a sublethal H_2O_2 concentration of 1.25 mM for 5 (T1) or 60 (T2) min. Twenty mL samples were collected by centrifugation at 5000 × *g* for 5 min and then washed twice with phosphate buffered saline. Membrane fatty acids were isolated from the pelleted cells according to the protocol of Sasser (1990) and identified using gas chromatography as described previously (Oberg et al., 2012). An untreated control sample was also prepared. Amounts of individual fatty acids were calculated as a percent of total and a two-tailed student t-test was used to determine differences in means between samples.

3. Results and discussion

3.1. Influence of H_2O_2 stress on global gene expression

To explore possible causes for differences in H_2O_2 resistance between *B. longum* NCC2705 and D2957, we analyzed their transcriptional changes after 5- or 60-min sublethal H_2O_2 exposure. Because our original DNA microarray design was based on the two *B. longum* genomes (NCC2705 and DJO10A) that were publicly available at the time, whole genome pyrosequencing of *B. longum* D2957 was first performed to better interpret the transcriptome results for this strain and to identify genes that were unlikely to hybridize to the microarray. Prior data from whole genome hybridizations with *Bifidobacterium* strains against the custom microarray (unpublished data) was used to estimate the cutoff threshold for hybridization of D2957 genes to the array. Those results suggested D2957 genes encoding proteins with ≤77%

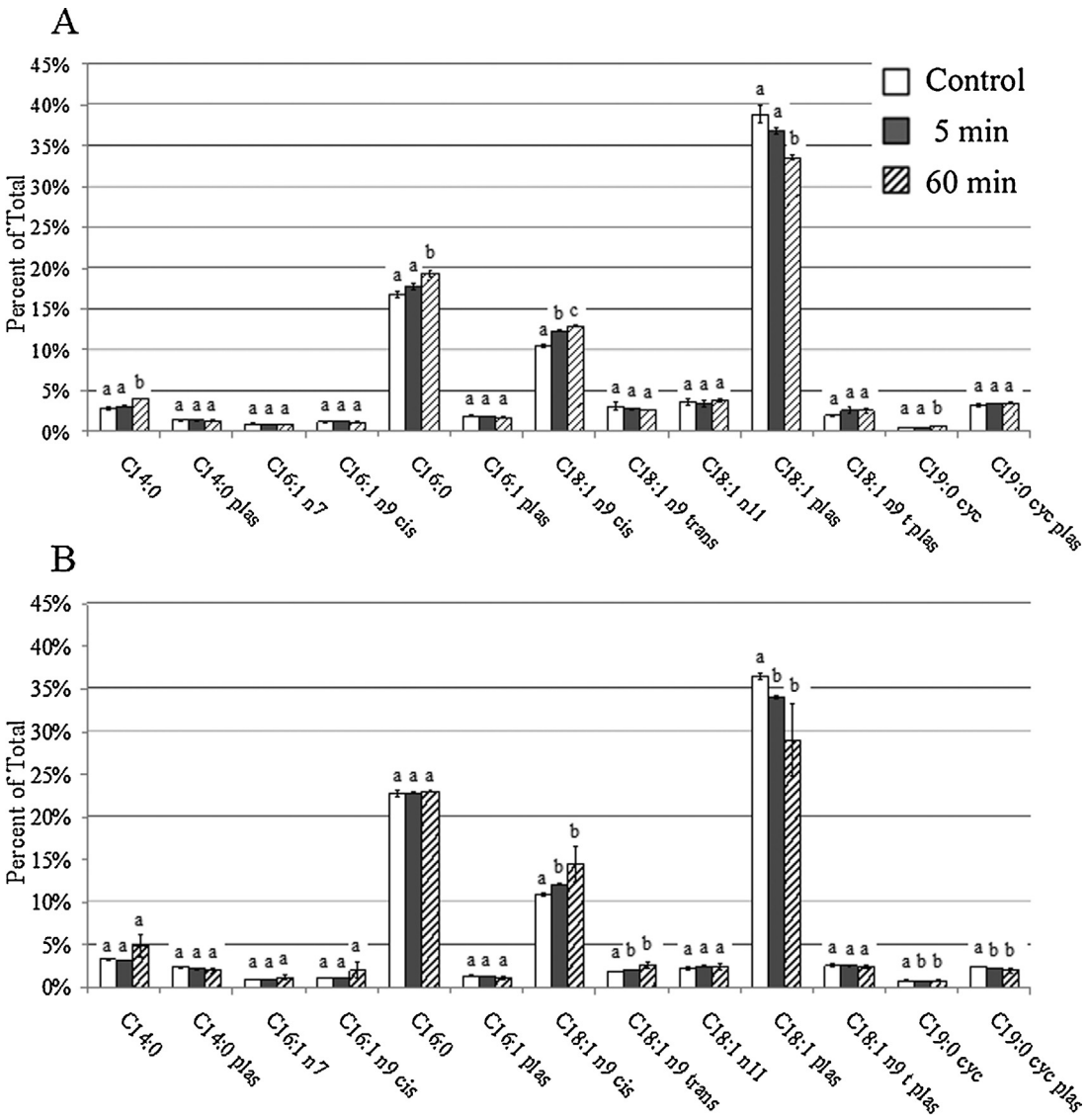

Fig. 3. Membrane fatty acid composition for *B. longum* NCC2705 (panel A) and *B. longum* D2957 (panel B). The graphs show data from cells grown in MP5 media with no H_2O_2 (open bar; "control") and cells exposed to 1.25 mM H_2O_2 in MP5 broth for 5 min (filled bar) or 60 min (hashed bar). Error bars correspond to the standard error of the mean (SEM). Means with the same letters within each strain are not statistically significantly different (p-value <0.05).

amino acid identity to array targets were unlikely to hybridize, and therefore deemed not detectable. Among the 2073 predicted open reading frames in the D2957 genome, 495 were below the cutoff value (see Supplementary material Table S1). The 350 (71%) of these genes encoded hypothetical proteins or had only general function.

Among the 1578 *B. longum* D2957 genes that were represented on the array, 21 showed statistically significant ($P < 0.05$) DE genes compared to control cells after 5 min exposure to a sublethal H_2O_2 exposure, and 90 differentially expressed (DE) genes were detected after 60 min exposure. Of these, 12 (60%) and 65 (76%) of the genes that were DE after 5 and 60 min, respectively, have an assigned function (Fig. 1 and Table S2). In contrast, *B. longum* NCC2705 showed a total of 288 significantly ($P < 0.05$) DE genes after a 5 min exposure, and 114 DE genes after 60 min (Fig. 1). Among the DE genes detected after 5 or 60 min, 192 (69%) and 83 (74%), respectively, have an assigned function (Fig. 2 and Table S2 in the Supplemental material).

RT-PCR analysis of 6 selected genes was used to validate microarray data obtained from *B. longum* NCC2705 and D2957. RT-PCR did not detect any contradictions between the two platforms (Fig. S1), and there was a positive correlation ($r^2 = 0.68$ and 0.63, respectively) between the fold-change for gene induction or repression predicted from the microarray and the respective values determined by RT-PCR.

Due to their anaerobic nature, *Bifidobacterium* ssp. lack the most common enzymes associated with oxidative stress defense, such as super oxide dismutase and catalase. However, other anaerobic bacteria have developed different systems to mitigate the toxic effects of H_2O_2 such as NADH peroxidases, peroxiredoxin, ferritine-like iron binding proteins and DNA repair enzymes (Yamamoto et al., 2011; Brioukhanov and Netrusov, 2007). Initial grouping of DE genes into predicted functional categories showed H_2O_2 exposure triggered up-regulation of genes involved in several such systems in both strains (Table 2). Examples include several genes involved in the thioredoxin reductase system which, under favorable growth conditions, functions with ribonucleoside reductase to use NADPH to reduce the 2′ OH group of ribose for deoxynucleotide production, as well as to maintain cytoplasmic redox for disulfide bond production in proteins (Nordlund and Reichard, 2006; Arnér and Holmgren, 2001). During oxidative stress; however, cells may use thioredoxin reductase and peroxiredoxin to direct NADPH toward the removal of oxidative free radicals via the reduction

of H_2O_2 and toxic lipid hydroperoxides (Reott et al., 2009; Meyer et al., 2009; Holmgren, 1989). Schell et al. (2002) suggested these enzymes might be one of the primary defense mechanisms against oxidative stress in bifidobacteria, and other research has shown up-regulation of thioredoxin, thioredoxin reductase and peroxiredoxin in response to oxygen stress (Ruiz et al., 2012; Xiao et al., 2011). *B. longum* NCC2705 showed significant up regulation of thioredoxin reductase (BL0614) and peroxiredoxin (BL0615) along with ribonucleotide reductase alpha and beta subunits (BL0670 and BL0671) after only 5 min sublethal H_2O_2 treatment (Table 2). In contrast, D2957 did not display significant upregulation of these genes until 60 min exposure to H_2O_2. These results confirm that thioredoxin reductase and peroxiredoxin provide a primary defense mechanism against oxidative stress in *B. longum* and suggest that the greater H_2O_2 resistance of strain NCC2705 versus D2957 may be tied to earlier induction of these mechanisms.

Oxidative stress causes protein damage and denaturation, and protection of proteins and processing of damaged proteins is an important component of the oxidative stress response in bacteria (Shacter, 2000; Stadtman and Levine, 2000; Cabiscol et al., 2000). Although, bifidobacteria possess well studied chaperones and chaperonins (e.g., DnaJ/DnaK and GroEL/GroES), their role in H_2O_2 resistance is unclear. *B. longum* NCC2705 for example, showed significant down regulation of several general stress response genes, including DnaJ, UspA and several chaperone proteins (BL0519, BL1250 and BL1664) in response to H_2O_2 exposure (Table 2). This observation is consistent with prior studies, which reported no change in expression or down regulation of the general stress response genes in response to acid stress (Jin et al., 2012; Sanchez et al., 2007). However, NCC2705 did show an up regulation of several proteases (BL0555, BL0781 and BL0945) after 5 min H_2O_2 exposure, including an ATP-dependent metallo-protease (BL1682). Jin et al. (2012) also reported upregulation of a similar metallo-protease during acid stress, which was hypothesized to function in response to damage of membrane proteins which result in perturbation of membrane function (Sakoh et al., 2005). In contrast, strain D2957 only showed upregulation of two proteases (BL0944 and BL0945) after 60 min exposure.

3.2. Membrane fatty acid analysis

Prior research we conducted on the oxidative stress responses of *B. animalis* subsp. *lactis* showed that two nearly genetically identical strains had statistically significant differences in bacterial cell membrane fatty acid composition (CMFA), and that these differences greatly affected the intrinsic resistance of those strains to H_2O_2 exposure (Oberg et al., 2012). These observations are consistent with other research that has shown changes in CMFA composition during stress exposure, and that modification of CMFA composition can increase cell resistance to environmental (Vigh et al., 2005; Zhang and Rock, 2008; Baysse and O'Gara, 2007; van Bokhorst-van de Veen et al., 2011; Cotter and Hill, 2003). Because of the prior observations in *Bifidobacterium*, the CMFA composition of *B. longum* strains NCC2705 and D2957 was determined after 5 min (T1) or 60 min (T2) 1.25 mM H_2O_2 exposure. Results showed that overall, NCC2705 had a significant increase in both C16:0 and C18:1 n9, but a decrease in the C18:1 plasmalogen when compared to cells grown in control media (Fig. 3). Strain D2957 showed a similar increase in C18:1 n9 and a decrease in C18:1 plasmalogen after exposure to 2.55 mM H_2O_2, but no change in C16:0 (Fig. 3).

Direct comparison between the two strains showed they maintain a similar membrane composition, with a very low saturated to unsaturated fatty acid ratio and that the majority of the unsaturated fatty acids occur as C18:1 plasmalogens (Oberg et al., 2012). These vinyl ether-linked lipids are more easily oxidized at the carbon oxygen ether bond, and do not propagate free radicals when oxidized compared to their ester linked counterparts (Catalá, 2009; Magnusson and Haraldsson, 2011). Interestingly both strains had low amounts of cyclopropyl fatty acids, which are suggested to decrease the amount of lipid peroxidation in cell membranes by stabilizing the unsaturated bond by addition of a methyl group (Pradenas et al., 2012).

4. Conclusions

In summary, transcriptome data suggest that the significant differences in the intrinsic and inducible resistance to H_2O_2 noted previously between *B. longum* NCC2705 and D2957 (Oberg et al., 2011) may be largely due to the timing and degree of induction of genes involved in an oxidative stress response. These data demonstrate that NCC2705 has a rapid and highly inducible H_2O_2 stress response, whereas strain D2957 showed a more delayed and less pronounced transcriptional response to H_2O_2 stress. Because of the highly reactive nature of oxidative free radicals, an immediate and robust inducible stress response may be more effective in neutralizing the damaging effects of H_2O_2.

Overall, these findings provide new insights to the molecular basis for oxidative stress resistance in *B. longum* and the factors that influence to strain-to-strain variability in survival in bioactive food products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.06.405.

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