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GENOME REPORTS

The complete genome sequence of *Bifidobacterium animalis* subsp. *lactis* 01 and its integral components of antioxidant defense system

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Abstract

The strain *Bifidobacterium animalis* 01, isolated from centenarians, showed promising antioxidant potential in our previous studies. In this study, the genome information on strain 01 and the important antioxidant components are presented. The complete genome comprises a single circular chromosome (1,931,632 bp; 60.49% G + C content) with 1569 coding DNA sequences, 52 tRNA, and 9 rRNA operons. Based on phylogenomic analyses, strain 01 was designated as *B. animalis* subsp. *lactis* 01. The genomic analysis reveals that at least eight protein-coding genes are antioxidant-related genes. The conditions for simulating the oxidative stress have been determined. The results of quantitative reverse transcription PCR further demonstrated that the genes encoding the thioredoxin system (*ahpC*, *ahpF*, *bcp*, *trxB*, *trxA*, *nrdH*, and *msrAB*) and non-enzyme factors of the divalent cation transporter gene (*mntH*) were upregulated under the H_2O_2 challenge, indicating that the eight genes were effective components of the antioxidant system. The results of this study could benefit for understanding the antioxidant mechanism of *B. animalis* 01 and future utilization of it as a potential antioxidant agent.

Keywords *Bifidobacterium animalis* subsp. *lactis* 01 · Complete genome sequence · Antioxidant activity · H_2O_2 challenge

Abbreviations

ROS	Reactive oxygen species
PCR	Polymerase chain reaction
RT-qPCR	Real-time quantitative PCR
COG	Clusters of orthologous groups
CFU	Colony-forming units
CDS	Coding DNA sequences
RAST	Rapid annotation using subsystem technology
GIT	Gastrointestinal tract
CICC	China Center of Industrial Culture Collection

Introduction

Reactive oxygen species (ROS) are produced by aerobic respiration and immune defense of organisms (Lü et al. 2010). The excessive amount of ROS can result in cellular damage, which promotes chronic diseases, such as cardiovascular diseases, diabetes, and cancer (Stephens et al. 2009; Sosa et al. 2013). The consumption of antioxidant supplements has been proposed to alleviate ROS and presumed beneficial for human health (Lobo et al. 2010). It has been shown that probiotic strains present significant antioxidant abilities (Mishra et al. 2015; Amaretti et al. 2013). In addition, certain probiotics can act as antioxidants to maintain intestinal redox balance in the gut by adhering to the intestinal lumen and colonizing the intestine (Tang et al. 2018).

A number of recent studies have reported that *Bifidobacterium* spp. showed great antioxidative activities (Mishra et al. 2015). The antioxidant capacity of *B. animalis* subsp. *lactis* INL1 was evaluated by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and ·OH technique. The different fractions, such as culture supernatant and lysate supernatant, exhibited high radical scavenging activity with both radicals (Loyeau et al. 2018). In *B. longum,* silent information regulator 2 could positively regulate the activity of its antioxidant enzymes (Guo et al. 2017). *B. bifidum* ATCC

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29521 could decrease the intracellular level of ROS, and its incubated extracts also showed antioxidative activity by chelating metal ions (Wang et al. 2016). *B.* subsp. *lactis* DSMZ 23032 showed an antioxidative capacity with total antioxidant activity, trolox equivalent antioxidant capacity, and total glutathione values (Amaretti et al. 2013). Therefore, these species are potential candidates of natural antioxidant bioresource to promote the human health.

B. animalis 01 was isolated from feces of healthy centenarian volunteers in Guangxi, China. *B. animalis* 01 itself and its protein extracted have been observed to possess antioxidant activity both in vitro and in vivo (Zhang et al. 2009; Shen et al. 2010, 2011). However, the antioxidant mechanism of this strain is still largely unknown. In this study, to further investigate its antioxidant mechanism, the complete genome information of strain 01 and its integral components of antioxidant defense system are presented. The results of this study lay the theoretical foundation for the future application of strain 01 in the prevention of oxidative stress-related disorders.

Methods

Genome sequencing, assembly, and annotation

The genomic DNA of strain 01 was extracted using the QIAGEN DNA Extraction Kit according to the manufacturer's instruction (Qiagen, CA, USA). Genome sequencing was performed by the Illumina Hiseq 2000 platform (2×100 bp). After sequencing, the short reads were assembled by SOAPdenovo v2.04 (http://soap.genomics.org.cn) (Luo et al. 2012).

After gap closing by SOAP GapCloser, a draft genome with 23 scaffolds was achieved. Gaps between scaffolds were closed by polymerase chain reaction (PCR) and Sanger sequencing. Genome annotation was applied by RAST (Overbeek et al. 2008). COG and Pfam (http://pfam.xfam.org/) were used to predict functional genes. The genes related to antioxidant activities were identified from the genome of *B. animalis* 01 using BLAST from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

H_2O_2 treatment of *B. animalis* subsp. *lactis* 01

As the most stable ROS, H_2O_2, which can diffuse through cells and form other active ROS, is generated from nearly all sources of oxidative cycle. Thus, H_2O_2 was added to the cultured strain 01 to induce the oxidative stress. Strain 01 was anaerobically cultured in De Man, Rogosa, and Sharpe (MRS) broth (AoBoxing, Beijing, China) at 37 °C until an OD600 of ~ 1.1, after which H_2O_2 was supplemented in a series (0.5, 1.0, 1.5, 2.0, and 2.5 mM), with the control containing no H_2O_2. The samples were collected at 0, 30, and 60 min after H_2O_2 addition and immediately diluted and plated on MRS with 0.05% cysteine agar. The plates were incubated anaerobically at 37 °C for 48 h before enumeration. The replicates were prepared in duplicate.

Determination of intracellular ROS

The production of intracellular ROS was measured using flow cytometry with 2′,7′-dichloro-uorescein diacetate (DCFH-DA) staining, as described previously (Li et al. 2017). After treatments with 1.5 mM H_2O_2 for 0, 30, and 60 min, cells were washed twice with phosphate buffer saline (PBS) (pH 7.2), and then, DCFH-DA (10 μM) was added to the cells for 30 min at 37 °C. The cells were washed with PBS to remove extracellular DCFH-DA and resuspended in PBS. The intracellular ROS levels were measured by FACSCalibur flow cytometry (BD Biosciences, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Real-time quantitative PCR (RT-qPCR) analysis

B. animalis subsp. *lactis* 01 was cultured as described above, after which H_2O_2 was supplemented at the selected concentration for 0 (T0), 5 (T1), 30 (T2), or 60 min (T3). The total RNAs of different samples were isolated using the Trizol reagent (Invitrogen, United States) following the manufacturer's instruction. To completely eliminate the DNA, the RNA samples were treated with 50 μg/mL RNase-free DNaseI (Takara, Japan). The quality of isolated RNAs was evaluated by gel electrophoresis. The RNA samples were reverse transcribed into single-stranded cDNA using PrimeScript first-strand cDNA Synthesis Kit (Takara, Japan). RT-qPCR was performed using 7500 Fast Real-Time PCR system (Applied Biosystems) with SYBR FAST qPCR Kit (Kapa Biosystems, USA). The primers sequences for RT-qPCR are listed in Table S1. The qPCR data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). And the 16S rRNA gene was used as an internal reference. Table S1 lists the gene-specific primer sequences.

Availability of data and materials

The complete genome sequence of *B. animalis* subsp. *lactis* 01 was deposited at GenBank under the accession number CP035497. This strain has been deposited in China Center of Industrial Culture Collection under the accession number CICC no. 24193. The community metadata standards the "Minimal Information about any (X) Sequence" (MixS), which is shown in Table 1.

Table 1 The community metadata standards the "Minimal Information about any (X) Sequence" (MixS) of *Bifidobacterium animalis* subsp. *lactis* 01

Investigation			
Investigation type	investigation_type		BA
Project name	project_name		PRJNA516982 Bifidobacterium animalis strain:01
Environment			
Collection date	collection_date		Missing (before 2000)
Geographic location (latitude and longitude)	lat_lon		
Geographic location (country and/or sea, region)	geo_loc_name		China: Guangxi
Environment (biome)	biome		Homo sapiens
Environment (feature)	feature		Gut
Environment (material)	material		Feces
MIMS/MIENS extension			
Environmental package	env_package		Feces
Depth	depth	Meter	1
Elevation	elev	Meter	0
Nucleic acid sequence source			
Number of replicons	num_replicons		3
Reference for biomaterial	ref_biomaterial		https://doi.org/10.1016/j.anaerobe.2010.06.006; https://doi.org/10.1007/s00284-010-9827-7; https://doi.org/10.1016/j.foodchem.2008.12.006
Observed biotic relationship	biotic_relationship		Free-living and particle-associated
Trophic level	trophic_level		Heterotroph
Relationship to oxygen	rel_to_oxygen		Anaerobes
Isolation and growth condition	isol_growth_condt		*B. animalis* subsp. *lactis* 01 grows optimally at a range of 37 °C, a pH of 6.5; culture media: MRS
Sequencing			
Sequencing method	sequencing_meth		Illumina
Assembly	assembly		Assembler: SOAPdenovo v2.04
Finishing strategy	finishing_strategy		Statuts: finished;
Relevant electronic resources	url		http://www.ncbi.nlm.nih.gov/genomeprj/CP035497

Results and discussion

General features

The complete genome of *B. animalis* 01 contains a circular chromosome of 1,931,632 bp and no plasmid, with G + C contents of 60.49%. The chromosome contains 1569 protein-coding genes, 52 tRNA, and 9 rRNA genes (Fig. 1). The identified genes were classified into 21 functional categories according to Clusters of Orthologous Groups (COG) of protein designation (Tatusov et al. 2003) (Table S2). Strain 01 was known as *B. animalis* 01 in our previous studies (Zhang et al. 2009; Shen et al. 2010, 2011). However, on the basis of the comparative phylogenomic analysis of *B. animalis* genomes, strain 01 should be now reassigned to denote *B. animalis* subsp. *lactis* 01 (Fig. S1).

Identification of gene-coding antioxidant system

According to the annotation of genome, eight antioxidant-related genes of strain 01 were identified (Table 2), included alkyl hydroperoxide reductase subunits C and F (*ahpC* and *ahpF*), bacterioferritin comigratory proteins (*bcp*), thioredoxin reductase (*trxB*), thioredoxin (*trxA*), glutaredoxin-like proteins (*nrdH*), peptide methionine sulfoxide reductase (*msrAB*), and divalent metal cation transporter (*mntH*). Among these genes, *ahpC*, *bcp*, *ahpF*, *trxB*, *trxA*, *nrdH*, and *msrAB* are the basic components of the thioredoxin (Trx) system, which is a functional antioxidant system in protecting cells from oxidative damage (Lu and Holmgren 2014). *MntH* actively acquires a high-affinity manganese. The involvement of manganese is critical for defensing against ROS (Huang et al. 2017).

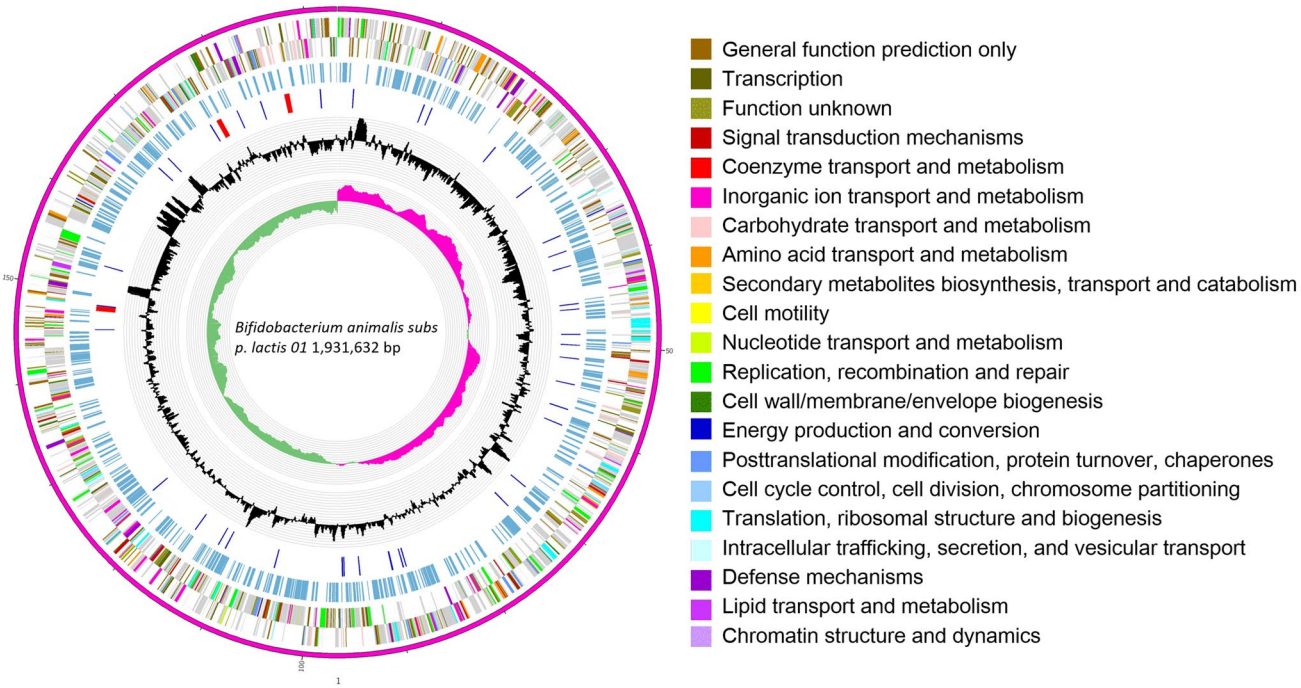

Fig. 1 Circular genome map of *Bifidobacterium animalis* subsp. *lactis* 01. Circles are shown from the outside to inner. Ring 1, Genome sequences. Ring 2 and 3, COG annotated coding sequences. Ring 4, KEGG enzymes. Ring 5, RNA genes. Ring 6, GC content. Ring 7, GC skew. Very short features were enlarged to enhance visibility. Clustered genes, such as several rRNA genes, may appear as one line due to space limitations. The image was created by software Circos

Table 2 Antioxidant-related genes in the *B. animalis* subsp. *lactis* 01 genome

COG category	Proposed function	Gene name	Locus tag(s)	Coordinates[c]	Protein size
O[a]	Alkyl hydroperoxide reductase subunit C	*ahpC*	ET527_04440	994497..995060(+)	187aa
O	Alkyl hydroperoxide reductase protein F	*ahpF*	ET527_04445	995079..996851(+)	590aa
O	bacterioferritin comigratory proteins	*bcp*	ET527_04555	1021161..1021646(+)	161aa
O	Thioredoxin reductase	*trxB*	ET527_08195	1923658..1924620(+)	320aa
O	Thioredoxin	*trxA*	ET527_06980	1609487..1609882(+)	131aa
O	Glutaredoxin-like protein	*nrdH*	ET527_01755	409364..409627(−)	87aa
O	Peptide methionine sulfoxide reductase *msrA*/*msrB*	*msrAB*	ET527_05320	1193250..1194242(−)	330aa
P[b]	Divalent metal cation transporter *mntH*	*mntH*	ET527_07110	1640760..1642118(−)	452aa

[a]Post-translational modification, protein turnover, chaperones

[b]Inorganic ion transport and metabolism

[c]Genes on forward strand (+); genes on reverse strand (−)

Resistance to H_2O_2

As shown in Fig. 2a, the datas of lgCFU were used to select appropriate H_2O_2 concentrations for oxidative stress induction and H_2O_2 challenge. The increased exogenous levels of H_2O_2 resulted in growth defect of strain 01. The strain 01 could survive well during the 1.0 mM of H_2O_2 challenge, indicating that these levels of H_2O_2 caused no metabolic disturbances which could impact the normal growth. The growth was stopped at a critical level of 1.5 mM of H_2O_2, which indicated a non-lethal stress. Significant cell death after the 60 min treatment with 2.0 mM of H_2O_2 was found. From Fig. 2b, c, we could also find the increase of intracellular ROS concentration in strain 01 treated by 1.5 mM of H_2O_2 with time variation. Therefore, 1.5 mM of H_2O_2 was selected for the oxidative stress induction and H_2O_2 challenge.

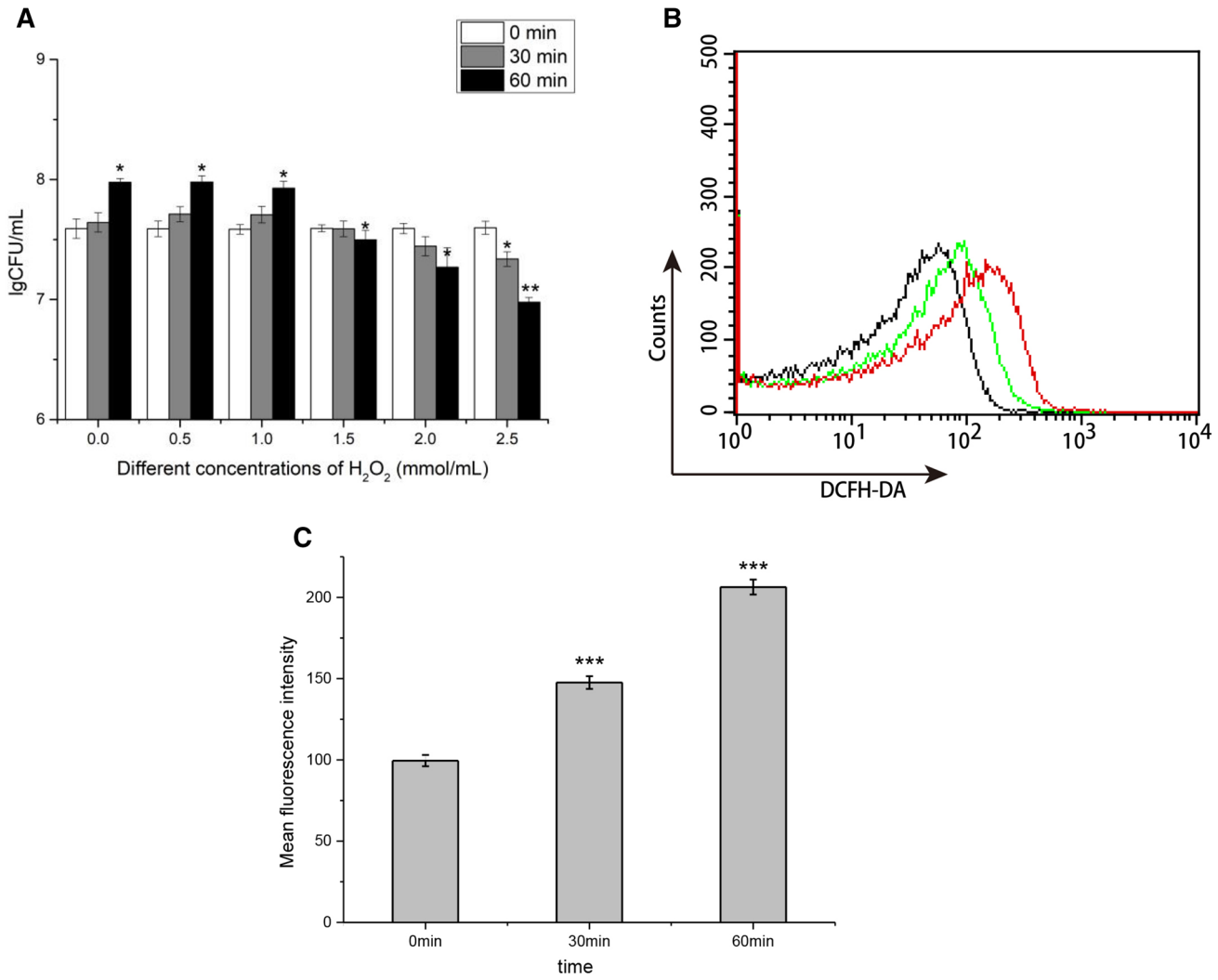

Fig. 2 Effect of H_2O_2 on survival and production of ROS in *B. animalis* subsp. *lactis* 01. **a** Survival of early exponential phase *B. animalis* subsp. *lactis* 01 over 30 min and 60 min in MRS broth medium with H_2O_2 concentration of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM. **b** Representative histograms of DCFH-DA fluorescence. Black line represented control *B. animalis* subsp. *lactis* 01 cells without H_2O_2 treatment (0 min). Green line represented *B. animalis* subsp. *lactis* 01 cells treated with 1.5 mM H_2O_2 (30 min), while red line represented cells treated with 1.5 mM H_2O_2 (60 min). **c** The percentage of mean fluorescence intensity relative to control cells. Data presented are the mean ± SD ($n = 3$). Error bars represent standard deviations. Statistical significance was calculated using Holm–Sidak Student's *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

Evaluation of the expression profiles of the antioxidant-related genes during H_2O_2 stress

As shown in Fig. 3, the transcription rates of eight antioxidant-related genes in strain 01 were elevated by 1.5 mM of H_2O_2. The gene expressions at T1 were higher than those at T2 and T3, indicating that strain 01 exhibited antioxidant activity with high values by scavenging H_2O_2 within minutes. The *ahpC* showed 5.3-fold upregulated expression, and the expression of *ahp*F increased by 3.35-fold after 5 min of oxidative stress exposure (T1). The trend of other relative gene expressions (T1) in the thioredoxin system was as follows: *trxB* > *bcp* > *trxA* > *msrAB* > *nrdH*. The expression of *mnt*H was upregulated by 5.46-fold after 5 min of oxidative stress exposure.

AhpC is a typical peroxiredoxin which is directly regenerated by AhpF through electron transfer. The ahpC and ahpF complex, which is widely distributed in prokaryotes, is known to efficiently detoxify H_2O_2 (Poole et al. 2000; Seaver and Imlay 2001). The ahpC-overexpressing *B. longum* subsp. *longum* NCC2705 showed increased resistance to the endogenous H_2O_2 versus the control strain (Zuo et al. 2014). Other studies have shown the upregulation of *ahpC* in *B. longum* subsp. *longum* BBMN68 and *B. animalis* subsp. *lactis* BL-04 under oxidative challenge (Oberg et al. 2013; Xiao et al. 2011). In *Salmonella typhimurium*,

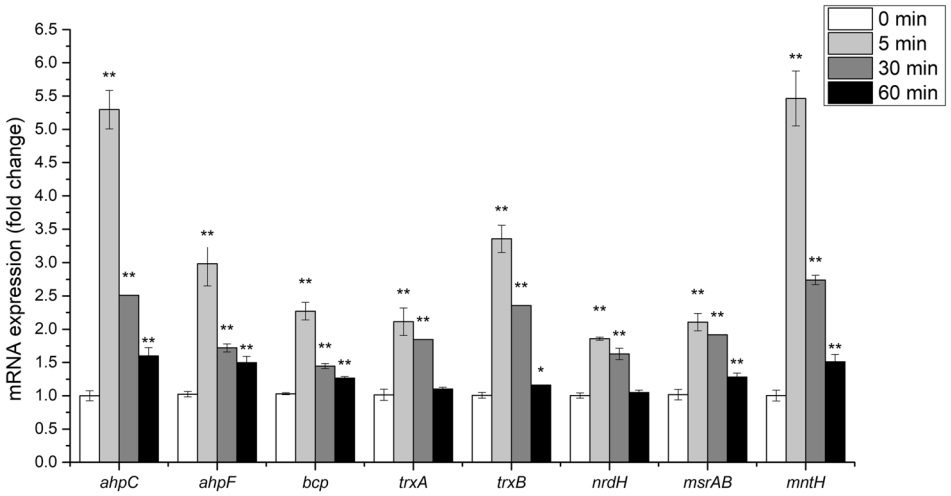

Fig. 3 Effects of hydrogen peroxide on expressions of antioxidant-related genes in *B. animalis* subsp. *lactis* 01. The bar chart showed the relative mRNA levels of eight tested genes of cells in the presence of 1.5 mM H_2O_2 at the early exponential phase. Data presented are the mean ± SD ($n = 3$). Error bars represent standard deviations. Statistical significance was calculated using Holm–Sidak Student's *t* test (*$p < 0.05$ and **$p < 0.01$)

ahpC is classified as a highly catalytic 2-Cys Prx, featuring a catalytic rate of more than 10^7/M/s (Parsonage et al. 2008). These evidences indicate that the ensemble of ahpC and ahpF is one of the most important antioxidant proteins in strain 01.

The thioredoxin system plays a crucial role in defensing against ROS for anaerobes (Jean et al. 2004; Mishra and Imlay 2013). Besides the AhpC and AhpF complex, other antioxidant proteins may also play important roles. Bcp, a member of peroxiredoxin, exhibits hydroperoxide peroxidase activities (Jeong et al. 2000). Poorly defined in *Bifidobacterium* sp., *bcp* was characterized with antioxidant functions from *Candidatus liberibacter asiaticus* and *Thermococcus kodakaraensis* KOD1 (Singh et al. 2017; Lee et al. 2015). The methionine residues are easily oxidized by ROS. Methionine sulfoxide reductase AB (*msrAB*) can catalyze the methionine sulfoxides back to methionine, and the cyclic interconversion of methionine can lead to the removal of ROS (Lee et al. 2009). Strain 01 showed significant upregulation of *trxB* and *trxA*, along with *nrdH*. The three genes were predicted for regenerating *bcp* and oxidizing *msrAB* by electron donation in response to the oxidative stress in strain 01.

Manganese has been proven beneficial for defensing against oxidative stress, acting as a co-factor of antioxidant enzymes and non-proteinaceous manganese antioxidants (Wang et al. 2014). The acquisition of manganese is crucial for cell manganese homeostasis. *MntH*, a high-affinity transporter, is highly selective for manganese (Kehres and Maguire 2003). The ability to obtain manganese plays an important role in catalase-void bacteria (Turner et al. 2015). To effectively cope with oxidative stress, strain 01 enhanced manganese import through the increased expression levels of *mntH*.

Conclusions

Unsurprisingly, anaerobic bacteria would exhibit antioxidant activity. The antioxidant activity is important for the survival of gut microbiota in GIT filled with ROS. Strain 01 lacks the most common antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. The antioxidant capacities of strain 01 may be associated with the thioredoxin system and manganese. Our study provides the transcriptional landscape of strain 01 under H_2O_2 challenge, and the results are highly meaningful for understanding the molecular mechanisms of ROS resistance in *Bifidobacterium* sp.

Author contributions JLZ and PLL designed the experiments. JLZ and SBW performed the experiments. JLZ, ZZ, SBW, and YXQ analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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