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Killed *Bifidobacterium longum* enhanced stress tolerance and prolonged life span of *Caenorhabditis elegans* via DAF-16

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Abstract

Probiotics are bacteria among the intestinal flora that are beneficial for human health. *Bifidobacterium longum* (BL) is a prototypical probiotic that is widely used in yogurt making, supplements and others. Although various physiological effects of BL have been reported, those associated with longevity and anti-ageing still remain elusive. Here we aimed to elucidate the physiological effects of killed BL (BR-108) on stress tolerance and longevity of *Caenorhabditis elegans* and their mechanisms. Worms fed killed BL in addition to *Escherichia coli* (OP50) displayed reduced body length in a BL dose-dependent manner. When compared with those fed *E. coli* alone, these worms had a higher survival rate following heat stress at 35°C and hydrogen peroxide-induced oxidative stress. A general decrease in motility was observed over time in all worms; however, killed BL-fed ageing worms displayed increased movement and longer life span than those fed *E. coli* alone. However, the longevity effect was suppressed in *sir-2.1*, *daf-16* and *skn-1*-deficient worms. Killed BL induced DAF-16 nuclear localisation and increased the expression of the DAF-16 target gene *hsp-12.6*. These results revealed that the physiological effects of killed BL in *C. elegans* were mediated by DAF-16 activation. These findings contradict previous observations with different *Bifidobacterium* and *Lactobacillus* strains, which showed the role for SKN-1 independently of DAF-16.

Key words: *Bifidobacterium longum*: Killed bacteria: BR-108: Longevity: Stress tolerance: *daf-16*

Caenorhabditis elegans feeds on *Escherichia coli* as food and lives for approximately 1 month on a nematode growth medium (NGM) plate. *C. elegans* is a commonly used model organism owing to its transparent body and ease of culture[1]. In addition, its short life span and genetic similarity with higher animals renders it suitable for studying longevity and as a model organism for various biological assays. Furthermore, its genome and cell lineages are well described. Lately, it has been used to evaluate the effect of certain food and their related functional components, including the anti-ageing effect of nucleoprotein extracted from salmon milt[2], longevity and stress tolerance effects of catalpol extracted from rehmannia roots[3], longevity effect of oreanolic acid[4] and others by feeding them along with *E. coli* to *C. elegans*.

The first longevity genes described in budding yeast, *C. elegans* and Drosophila were the sirtuin genes. Longevity genes such as *SIR2* and *TOR1* of budding yeast[5,6]; *sir-2.1, daf-16* and *age-1* of *C. elegans*[7,8]; and *Sir2* and *dFOXO* of Drosophila[9] are well described. These genes mediate longevity[10–12], lower the reactive oxygen species (ROS) level[3,13], suppress ageing[2] and increase stress tolerance[3,4]. For example, activated DAF-16 translocates into the nucleus and acts as a transcription factor[14] for genes related to stress tolerance, such as *sod-3*[15,16] and *hsp-12.6*[17]. Up-regulation of these genes prolongs the life span of the nematode and increases stress tolerance[11,18]. Conversely, deficiency of these genes shortens the life span and decreases stress tolerance[19,20]. Longevity genes are also found in higher animals. For example, humans possess approximately fifty members of the FOX transcription factors[21,22]. *FoxO*, a member of the FOX transcription factor family, is the homologue of the *C. elegans daf-16*[23] and Drosophila *dFOXO*[24].

In 1908, Metchnikoff first described that Bulgarians ingesting *Lactobacillus* regularly by consuming yogurt showed longevity[25,26]. This is the first description of the association between longevity and probiotics. *Bifidobacterium* is a well-known probiotic and constitutes the majority of the intestinal flora in infants[27]. Although the proportion is reduced after infancy, the number of *Bifidobacterium longum* (BL) in the intestine is 100–1000 times higher than that of *Lactobacillus*[27], and BL is known to promote the health of the host. Recent studies have described several physiological effects of BL, such as to regulate intestinal function and its beneficial effects in immunity and allergy reduction[28,29]. Furthermore, the role of *Bifidobacterium* in suppression of cancer has been reported[30].

Abbreviations: BL, *Bifidobacterium longum*; ROS, reactive oxygen species.

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Despite the potential benefits of *Bifidobacterium* mentioned above, its physiological effects on longevity and anti-ageing and their mechanisms are not well described. Here, we evaluated the effects of heat-killed BL (BR-108). Killed bacteria were used in this study, considering bacteria ingested by humans are likely to be killed by digestive juices. We aimed to analyse the physiological effects of BL on stress tolerance, anti-ageing and longevity in *C. elegans* and their mechanisms.

Methods

Nematode and Bifidobacterium

C. elegans strains used in this study were as follows: N2 Bristol (wild type), *daf-16 (mgDf50)*, *sir-2.1 (ok434)*, *skn-1 (tm4241)*, *col-19::GFP (TP12)*, *daf-16::GFP (TJ356)* (obtained from the Caenorhabditis Genetics Center, University of Minnesota) and *skn-1 (tm4241)* (obtained from the National BioResource Project, Tokyo Women's Medical University). The nematodes were cultured at 20°C on NGM plates spread with *E. coli* (OP50)[1]. BL (BR-108) sterilised at 105°C for 20 min was obtained from the Combi Corporation.

Nematode synchronisation

To collect eggs, adult nematodes were crushed in NaClO solution (1:10 of 10 M NaOH (Wako Pure Chemical Industries, Ltd) and NaClO (Haiter; KAO)). This method was used to synchronise the nematode growth level.

Body length measurement

Synchronised worms were placed on NGM plates spread with OP50 only (OP plates) or along with BL at various concentrations (BL plates; 1·0, 2·0, 3·0 or 5·0 mg/ml) and cultured at 20°C for 96 h. Thereafter, the worms were fixed with 10 % ethanol (Kanto Chemical Co., Inc.) and observed under a BZ8000 microscope (Keyence Corporation). Body length was determined using the ImageJ software (NIH). The body length of the control worms was set as 100 % and >30 worms were evaluated per group.

Measurement of COL-19 expression

COL-19 expression was used as the indicator for ageing, and adult *col-19::GFP (TP12)* worms expressing fluorescent COL-19 protein were used in the experiment. Synchronised *TP12* worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) at 20° C for 52 h. Thereafter, the worms were fixed with 10 % ethanol and observed under a BZ8000 microscope. The fluorescence was analysed using the ImageJ software. The fluorescence of control worms was set as 100 % and >25 worms were evaluated per group.

Evaluation of nematode motility

The movement of wild-type, *MgDf50* and *tm4241* worms was evaluated. Synchronised worms were cultured on OP plates for 96 h and then transferred to OP or BL plates (0·5 or 5·0 mg/ml). The day of the initial transfer to OP or BL plates was designated as day 0. The worms were then transferred to new plates every 3 d and thrashing movement of the worms was counted on each transfer day. To prevent offspring generation, 0·5 mg/ml FUdR (2'-deoxy-5-fluorouridine; Wako Pure Chemical Industries, Ltd) was added into the plates at days −1, 0 and 3. The movement at day 0 was set as 100 % and 10 worms were evaluated per group.

Assessment of heat stress tolerance

Synchronised worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) at 20°C for 96 h and then incubated at 37°C for 3·5 h. The day of heat application was designated as day 0. As described above, 0·5 mg/ml FUdR was applied to the plates at days −1 and 0. The survival rate of thirty worms/group was evaluated every 2 d.

Generally, heat stress decreased the worm movement. To measure the recovery rate from heat stress, synchronised worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) for 96 h and then transferred to OP plates and incubated at 35°C for 4 h. The time of heat application was designated as the 0 h, and thrashing movement was counted every 12 h. In parallel, the movement of worms kept at 20°C instead of 35°C was counted. The ratio of movement count of worms cultured at 35°C and 20°C was calculated and the results are presented. The movement of ten worms/group was counted.

Table 1. Sequences of primers used in the gene expression analysis

Genes	5'–3' Sense	5'–3' Anti-sense
Actin	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA
daf-16	ATCATCTTTCCGTCCCCG	TTGGAATTGCTGGAACCG
sod-3	GCTGCAATCTACTGCTCGCACTG	GGCTGATTACAGGTTCCAAATCTGC
hsp-12.6	TGGAGTTGTCAATGTCCTCG	GACTTCAATCTCTTTTGGGAGG
hsp-16.2	TGTTGGTGCAGTTGCTTCGAATC	TTCTCTTCGACGATTGCCTGTTG
hsp-70	ACCCTTCGTTGGATGGAACG	GCATCCGGAACCTGATTGGGC
rab-10	TATTACCGCGGAGCAATGGG	TCTTCCGATGCATGTTCGTCA
skn-1	TTCAAACTCCCACCCGAATGT	TGGTGTTGGTGGTGGTAGAG
ins-39	CGTCCGACTTCATCCCTCAC	GCTGAGCAGAAGACCAGGAG
sod-1	TGGTGGACCAAAATCCGAGA	CCATAGATCGGCCAACGACA
pha-4	CTCGGCCGCCAACCTATAAA	CGGTTGAAACCAATGGCAGG
myo-2	CCCTCGATCGTCAGACACAG	GGAGTCATATGCGCGGGATT
ctl-1	GTCGTTCATGCCAAGGGAG	ACGAACGAGAAGTGGTGTCT
ctl-2	TCCCAGATGGGTACCGTCAT	AAGTTGACCGGCCTTCTCC

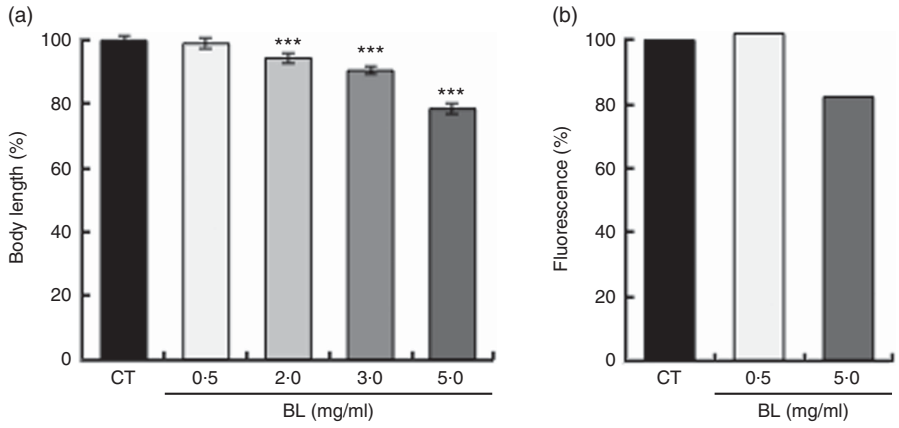

Fig. 1. Effects of *Bifidobacterium longum* (BL) on worm growth and development. (a) Synchronised worms were placed on plates containing *Escherichia coli* (OP50) alone (OP plates; CT) or with different concentrations of killed BL (1·0, 2·0, 3·0 or 5·0 mg/ml). Worms were cultured on these plates for 96 h. (b) Synchronised *TP12* worms were cultured on OP (CT) or BL plates (0·5 or 5·0 mg/ml) for 52 h. In both assays, the worms were fixed in 10 % ethanol and observed under a microscope to assess body length and fluorescence. The body length and fluorescence of control worms were set as 100 %. Values are means ($n > 30$ and $n > 25$ for the two assays, respectively), with their standard errors represented by vertical bars. *** $P < 0{\cdot}005$ by Tukey's *t* test.

In addition to that in wild-type worms, the recovery from heat stress in *mgDf50* and *tm4211* mutant worms was also evaluated using the method described above. The worms were cultured on OP or BL plates (5·0 mg/ml), and thrashing movement of *mgDf50* and tm4211 worms was counted every 12 and 6 h, respectively. The movement of 10 worms/group was counted.

Evaluation of oxidative stress tolerance

Synchronised worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) for 96 h and then transferred to 500 µl of 0·3 % H_2O_2 (Sigma-Aldrich Japan) in a 24-well plate (Techno Plastic Products AG). The time of transfer was designated as the 0 h. The worm survival rate was determined every hour starting from 2 h after the transfer. The survival rate at 0 h was set as 100 %, and 24 worms were assessed per group.

Tolerance to oxidative stress in *mgDf50* and *tm4211* worms was also evaluated using the method described above. The worms were cultured on OP or BL plates (5·0 mg/ml) and 24 worms were assessed per group.

Evaluation of life span

Synchronised worms were cultured on OP plates for 96 h and then transferred to OP or BL plates (5·0 mg/ml). The day of the initial transfer to OP or BL plates was designated as day 0. The worms were then transferred to new plates every 2 d. Alive and dead worms were counted on each transfer day. Worms that displayed no movement upon gentle probing with a platinum picker were judged as dead. As described above, 0·5 mg/ml FUdR was added into the plates at days −1, 0, 2 and 4. In addition to that of wild-type worms, the life span of *MgDf50*, *ok434* and *tm4241* worms was also assessed. The survival rate of sixty worms/group was determined.

Evaluation of fat accumulation

Synchronised worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) for 96 h and then fixed with 4 % PFA (Wako Pure Chemical Industries, Ltd) at 4°C. Fixed worms were washed and

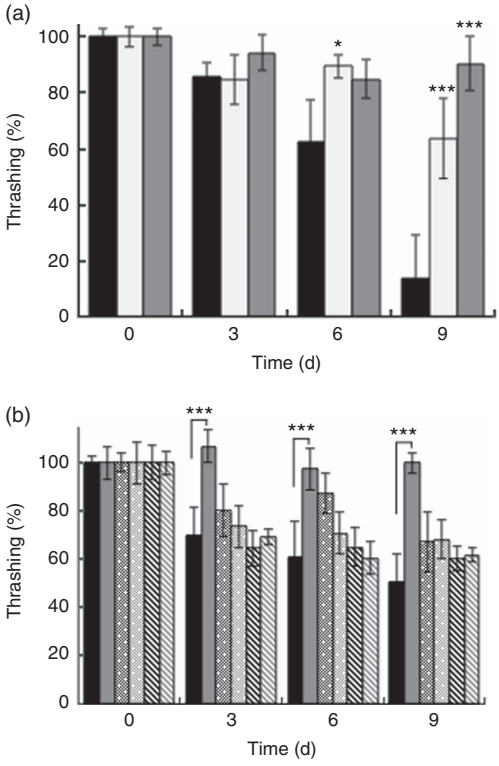

Fig. 2. Effects of *Bifidobacterium longum* (BL) on worm movement. Synchronised (a) N2 worms or (b) N2, *MgDf50* and *tm4241* worms were cultured on plates with *Escherichia coli* (OP50) (OP plates) for 96 h and then transferred to OP plates (CT) or plates with killed BL (a: 0·5 or 5·0 mg/ml; b: 5·0 mg/ml), which was designated as day 0. The worms were then transferred to new plates every 3 d and thrashing movement of the worms was counted on each transfer day. To prevent offspring generation, 0·5 mg/ml FUdR was added to the plates at days −1, 0 and 3. The movement count at day 0 was set as 100 %. Values are means (n 10 per group), with their standard errors represented by vertical bars. a: ■, CT; □, BL 0·5; ▩, BL 5·0 (mg/ml); b: ■, CT (N2); ▩, BL 5·0 (N2); ▩, CT (mgDf50); ▩, BL 5·0 (mgDf50); ▩, CT (tm4241); ▩, BL 5·0 (tm4241) (mg/ml). * $P < 0{\cdot}05$, *** $P < 0{\cdot}005$ by Tukey's *t* test.

then placed in plates containing 500 µl of 5 µg/ml Nile red (Wako Pure Chemical Industries, Ltd) for 10 min at 4°C. Subsequently, the worms were washed again twice and fluorescence was

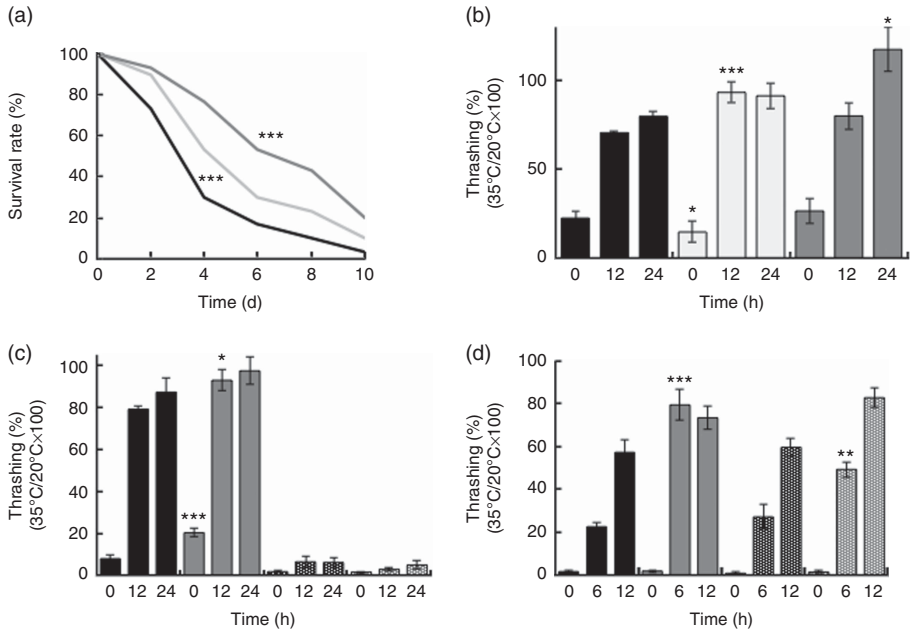

Fig. 3. Heat stress tolerance of worms fed *Bifidobacterium longum* (BL). (a) Synchronised wild-type N2 worms were cultured on plates with *Escherichia coli* (OP50) alone (OP plates; CT) or with killed BL (BL plates; 0·5 or 5·0 mg/ml) for 96 h and then heated at 37°C for 3·5 h, which was designated as day 0. To prevent offspring generation, 0·5 mg/ml FUdR was added into the plates at days −1 and 0. The worm survival was recorded every 2 d, *n* 30/group. *** $P<0·005$ by log-rank test. (b–d) Synchronised wild-type N2 worms, as well as *mgDf50* (c) and *tm4211* worms (d), were cultured on OP (CT) or BL plates (b, 0·5 or 5·0 mg/ml; c–d, 5·0 mg/ml) for 96 h and then transferred to OP plates and either left at 20°C or heated at 35°C for 4 h (the time of heat application was designated as 0 h). Thrashing movement was counted every 12 or 6 h. The ratio of movement count of worms cultured at 35°C and 20°C was calculated. Values are means (*n* 10 per group), with their standard errors represented by vertical bars. a: ——, CT; ——, BL 0·5; ——, BL 5·0 (mg/ml); b: ■, CT; □, BL 0·5; ▩, BL 5·0 (mg/ml); c: ■, CT (N2); ▩, BL 5·0 (N2); ▦, CT (mgDf50); ▨, BL 5·0 (mgDf50) (mg/ml); d: ■, CT (N2); ▩, BL 5·0 (N2); ▦, CT (tm4241); ▨, BL 5·0 (tm4241) (mg/ml). * $P<0·05$, ** $P<0·01$, *** $P<0·005$ by Tukey's *t* test.

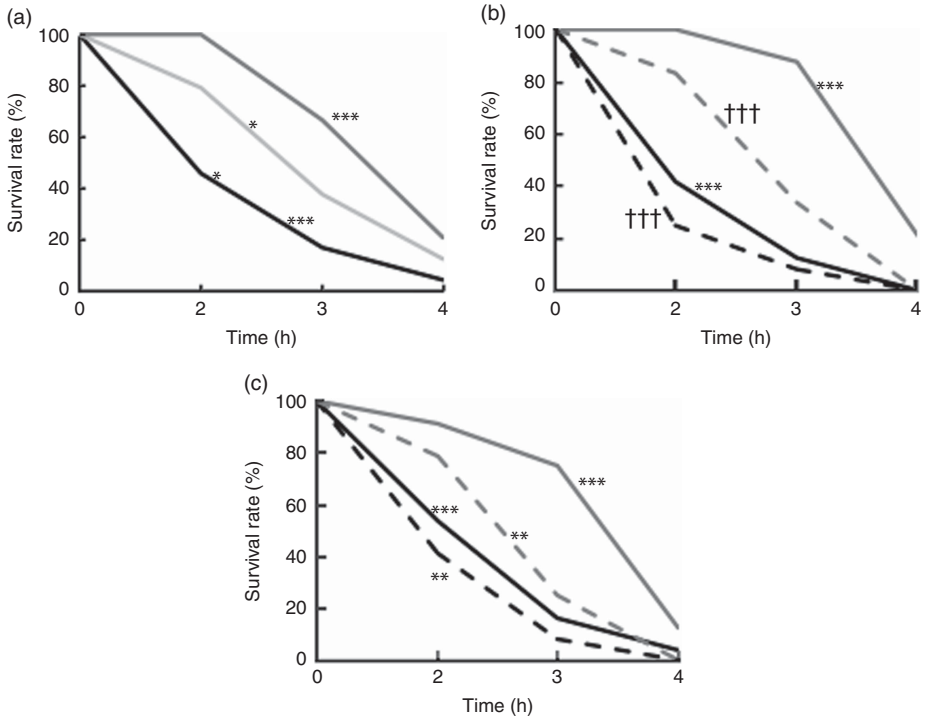

Fig. 4. Oxidative stress tolerance of worms fed *Bifidobacterium longum* (BL). Synchronised wild-type N2 (a–c), as well as *mgDf50* (b) and *tm4211*, worms (c) were cultured on plates with *Escherichia coli* (OP50) alone (OP plates; CT) or with killed BL (BL plates; a: 0·5 or 5·0 mg/ml; b–c: 5·0 mg/ml) for 96 h and then transferred to a 24-well plate with 500 μl of 0·3 % H_2O_2 (the time of worm transfer was designated as 0 h). The survival rate was assessed hourly starting at 2 h after the transfer. The survival rate at 0 h was set as 100 %. a: ——, CT; ——, BL 0·5; ——, BL 5·0 (mg/ml); b: ——, CT (N2); ——, BL 5·0 (N2) (mg/ml); – – –, CT (mgDf50); – – –, BL 5·0 (mgDf50) (mg/ml); c: ——, CT (N2); ——, BL 5·0 (N2); – – –, CT (tm4241); – – –, BL 5·0 (tm4241) (mg/ml). * $P<0·05$, ** $P<0·01$, *** $P<0·005$, ††† $P<0·005$ by log-rank test, *n* 24 per group.

captured using a BZ8000 microscope and analysed using the ImageJ software. The fluorescence of control worms was set as 100 %, and >35 worms were evaluated for each group.

Mitochondria activation assay

Synchronised worms were cultured on OP or BL plates (5·0 mg/ml) for 72 h. To evaluate the mitochondrial membrane potential and mitochondrial ROS, 0·5 mg/ml MitoTracker Orange CMTMRos (Thermo Fisher Scientific, Inc.) and 0·5 mg/ml MitoTracker Orange CM-H_2TMRos (Thermo Fisher Scientific) were added into the plates at a volume of 400 µl. After 24 h, the worms were washed and then fixed with 10 % ethanol. The fluorescence was captured using a BZ8000 microscope and analysed using the ImageJ software. The fluorescence level of control worms was set as 100 %, and >55 worms/group were analysed.

Cellular localisation of DAF-16

The cellular localisation of DAF-16 was assessed in *TJ356* worms. Synchronised worms were cultured on OP or BL plates (1·0, 2·0, 3·0 or 5·0 mg/ml) for 96 h and then fixed with 10 % ethanol. Fluorescence images were captured using a BZ8000 microscope and analysed using the ImageJ software. Activated DAF-16 could be observed as bright dots. DAF-16 cellular localisation pattern in the worms can be classified into the following types: nucleus, partially nucleus, and cytoplasmic. More than twenty worms were assessed per group.

Gene expression analysis

Synchronised worms were cultured on OP or BL plates (0·5 or 5·0 mg/ml) for 96 h. For mRNA extraction, the worms were crushed using the Power Masher II and Bio Masher II (Nippi, Inc.). The PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio Inc.) was used for the complementary DNA synthesis. Subsequently, quantitative PCR (qPCR) was performed using the Thunderbird SYBR qPCR Mix (Toyobo) and gene-specific primers (Table 1) in a Thermal Cycler Dice Real Time System Lite (Takara Bio Inc.) instrument. Actin was used as the reference gene, and each qPCR reaction was performed in triplicate wells.

Statistical analysis

Data are shown as the means with their standard errors. Tukey's test and log-rank test were used for the data analysis. Graphs were generated using the Microsoft Excel and PowerPoint software (Microsoft Corp.). P values <0·05 indicate statistically significant differences.

Results

Nematode growth and development

BL supplementation shortened the body length of the worms in a dose-dependent manner (Fig. 1(a)). As assessed by fluorescence, BL supplementation decreased the expression of COL-19 in *TP12* worms in a dose-dependent manner (Fig. 1(b)).

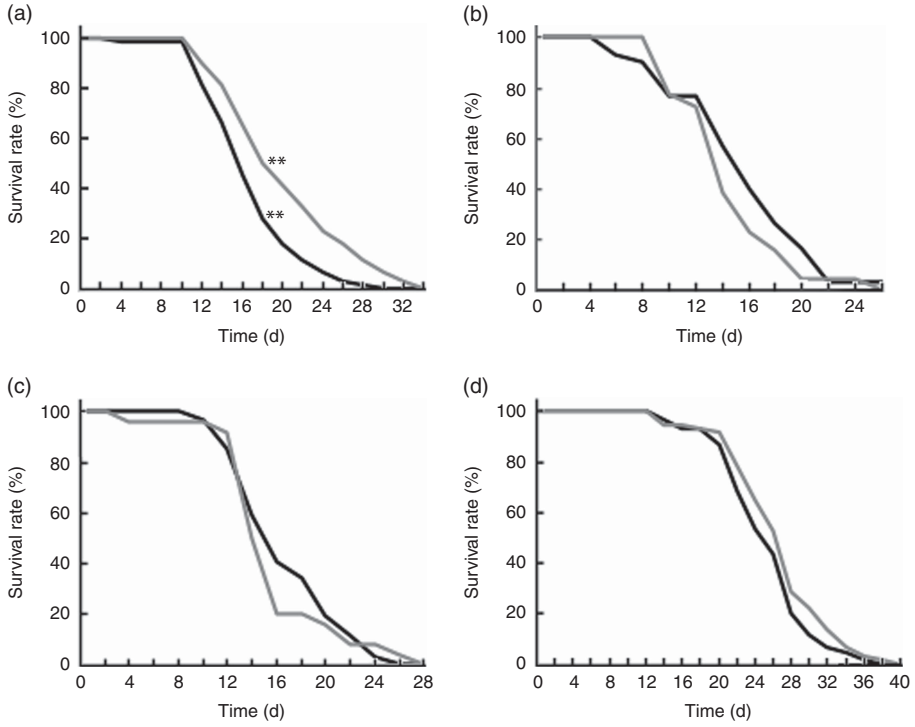

Fig. 5. Life span of worms fed *Bifidobacterium longum* (BL). Synchronised wild-type N2 (a), *MgDf50* (b), *ok434* (c) and *tm4241* worms (d) were cultured on plates with *Escherichia coli* (OP50) (OP plates) for 96 h and then transferred to a new OP plate (CT) or plates with killed BL (5·0 mg/ml), which was designated as day 0. The worms were transferred to new plates every 2 d, and the worm survival was assessed on each transfer day. To prevent offspring generation, 0·5 mg/ml FUdR was added into the plates at days −1, 0, 2 and 4. a: ——, CT; ——, BL 5·0 (mg/ml); b: ——, CT (mgDf50); ——, BL 5·0 (mgDf50) (mg/ml); c: ——, CT (ok434); ——, BL 5·0 (ok434) (mg/ml); d: ——, CT (tm4241); ——, BL 5·0 (tm4241) (mg/ml). ** P<0·01 by log-rank test, n 60 per group.

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Anti-ageing effects of Bifidobacterium longum

Generally, all worms displayed reduced motility over the course of the experiment. However, wild-type N2 worms fed killed BL displayed increased motility compared with control worms fed *E. coli* alone. At day 9, worms fed 0·5 mg/ml and 5 mg/ml BL showed a 6- and 9-fold higher average of thrashing movements, respectively, compared with those fed *E. coli* only (Fig. 2(a)). However, BL supplementation failed to conserve the movement of ageing *tm4241* worms, whereas *mgDf50* worms fed BL displayed reduced motility than those fed *E. coli* alone (Fig. 2(b)).

Bifidobacterium longum *increased the nematode tolerance to heat and oxidative stress*

Supplementation with killed BL in wild-type N2 worms improved the worm survival during heat stress in a dose-dependent manner (Fig. 3(a)). BL also increased the rate of recovery from heat stress (Fig. 3(b) and (d)) in wild-type N2 worms. Together, these findings suggested that BL increased tolerance to heat stress in *C. elegans*. However, BL did not increase the recovery rate of *mgDf50* worms (Fig. 3(c)), but increased that of *tm4241* worms (Fig. 3(d)).

Similarly, BL increased the survival rate during oxidative stress in a dose-dependent manner (Fig. 4(a)) in wild-type N2 worms. In contrast to the findings on heat stress, BL also increased the survival rate of both *mgDf50* (Fig. 4(b)) and *tm4241* worms (Fig. 4(c)).

Bifidobacterium longum *affected longevity, fat accumulation and mitochondrial activity*

Wild-type N2 worms fed killed BL showed significantly longer life span when compared with those fed *E. coli* only (Fig. 5(a)). However, BL did not improve the longevity of *mgDf50* (Fig. 5(b)), *ok434* (Fig. 5(c)) or *tm4241* worms (Fig. 5(d)).

BL also decreased the fat accumulation in N2 worms in a dose-dependent manner (Fig. 6(a)), and increased mitochondrial membrane potential (Fig. 6(b)) and mitochondrial ROS level (Fig. 6(c)) in N2 worms when compared with those observed in the control worms.

Bifidobacterium longum *affected cellular localisation of DAF-16 and expression of its target genes*

As shown by green fluorescence in *TJ356* worms, BL induced nuclear localisation of DAF-16 (Fig. 7(b) and (d)) when compared with that observed in *TJ356* control worms fed *E. coli* only (Fig. 7(a) and (c)). Further analyses showed that DAF-16 nuclear localisation was dependent on the dosage of BL (Fig. 7(e)).

Furthermore, qRT-PCR results showed that BL increased the level of *daf-16* mRNA and markedly increased the expression of one of its target genes – *hsp-12.6* (Fig. 8). Although to a lesser extent, BL also significantly increased the relative mRNA level of *hsp-16.2*, *hsp-70*, *skn-1*, *ctl-1*, *ctl-2* and *sod-1*.

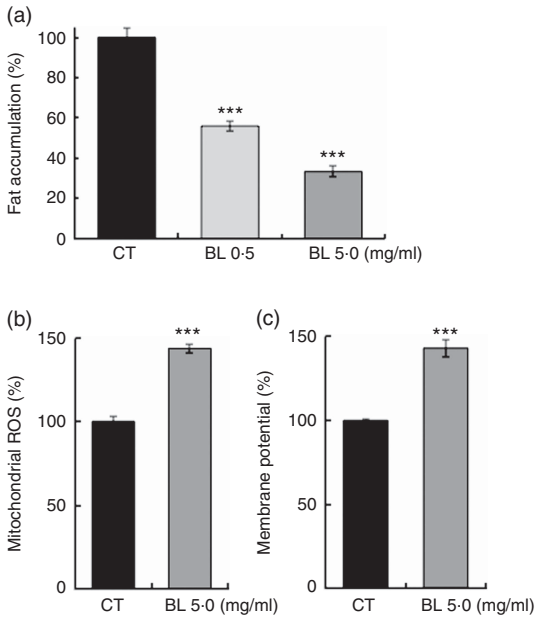

Fig. 6. Fat accumulation and mitochondria activation in worms fed *Bifidobacterium longum* (BL). (a) Synchronised wild-type N2 worms were cultured on plates with *Escherichia coli* (OP50) alone (OP plates; CT) or with killed BL (BL plates; 0·5 or 5·0 mg/ml) for 96 h. The worms were then fixed with 4 % PFA and stained with Nile red. (b, c) Synchronised worms were cultured on OP (CT) or BL plates (5·0 mg/ml) for 72 h, and 0·5 mg/ml MitoTracker Orange CMTMRos and 0·5 mg/ml MitoTracker Orange CM-H_2TMRos were added into the plates at a volume of 400 µl. After 24 h, the worms were washed and fixed with 10 % ethanol. In both assays, the fluorescence was measured and analysed using a BZ8000 microscope and the ImageJ software, respectively. The fluorescence of the control worms was set as 100 %. Values are means ($n > 35$ and $n > 55$ worms for the two assays, respectively), with their standard errors represented by vertical bars. ROS, reactive oxygen species. *** $P < 0{\cdot}005$ by Tukey's *t* test.

Discussion

The present study elucidated several physiological effects of killed BL in *C. elegans*, including enhanced stress tolerance, increased motility and prolonged life span. Feeding worms with BL affected the growth and development of the worms. As shown by the fluorescence of *col-19::GFP* (*TP12*) worms, those fed a high concentration of BL expressed a lower level of COL-19 protein, indicating higher larva ratio and delayed growth on these worms[31,32] without affecting reproduction (online Supplementary Fig. S1). This may be associated with shorter body length observed in worms fed BL (Fig. 1(b)). It is widely thought that stress tolerance, longevity and development are highly related. A previous report showed that stress tolerance and longer life span are typically observed in the event of delayed growth[33].

In this study, BL appeared to enhance tolerance to heat stress (Fig. 3(a)) and H_2O_2-induced stress in *C. elegans* (Fig. 4(a) and (c)) and conserved the motility in aged worms (Fig. 3). Although the motility conservation was not consistently observed throughout the experimental period, BL appeared to improve the worm motility in a dose-dependent manner. Furthermore, BL significantly prolonged the life span of the worms (Fig. 5(a)).

Nevertheless, BL failed to increase the heat stress tolerance in *mgDf50* worms (Fig. 3(c)) and did not prolong the life span of

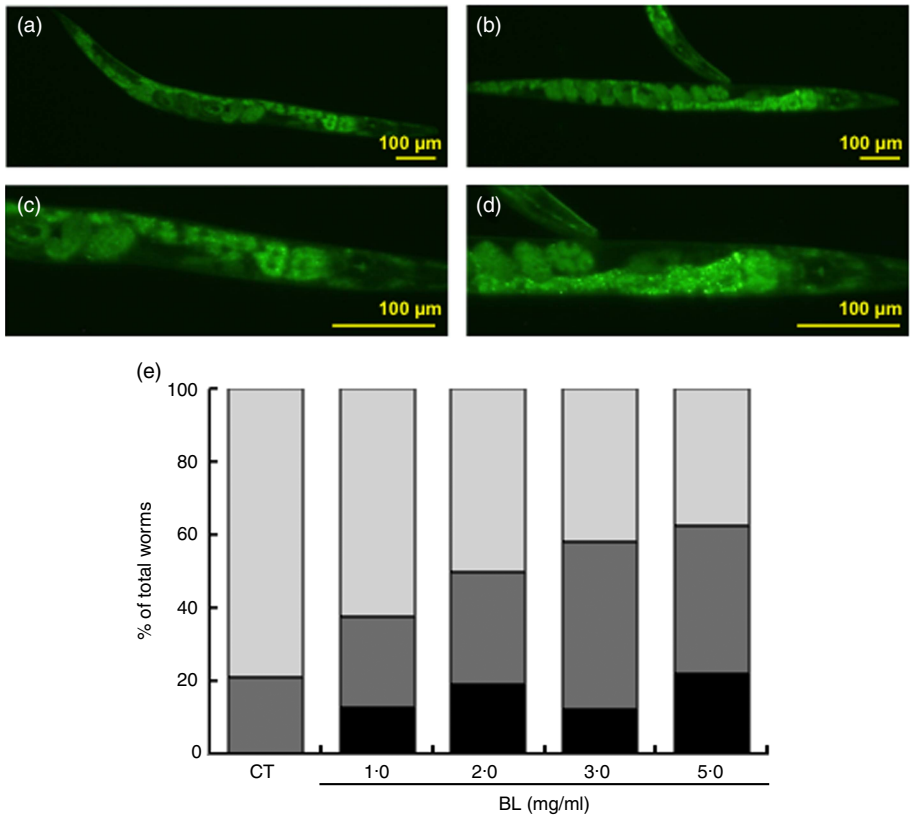

Fig. 7. Cellular localisation of DAF-16. The cellular localisation of DAF-16 was evaluated using *TJ356* worms. Synchronised worms were cultured on plates with *Escherichia coli* (OP50) alone (OP plates; CT) or with killed *Bifidobacterium longum* (BL plates; 1·0, 2·0, 3·0 or 5·0 mg/ml) for 96 h and then fixed with 10 % ethanol. Images were captured using a BZ8000 fluorescent microscope and analysed with the ImageJ software. Representative images of (a) control worms (CT) and (b) those given 5·0 mg/ml BL are shown. (c, d) Higher-magnification images of panels a and b, respectively. The scale bars indicate 100 μm. (e) DAF-16 cellular localisation was classified into three types – nuclear, partial nuclear and cytoplasmic – and the results are graphed and presented. e: ■, Nuclear; ■, partial nuclear; □, cytoplasmic.

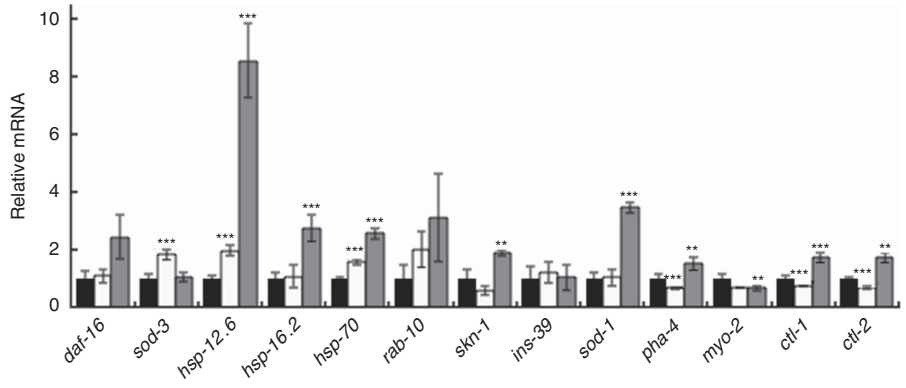

Fig. 8. Gene expression analysis of worms fed *Bifidobacterium longum* (BL). Synchronised worms were cultured on plates with *Escherichia coli* (OP50) alone (OP plates; CT) or with killed BL (BL plates; 0·5 or 5·0 mg/ml) for 96 h. qRT-PCR analysis was performed using the Thermal Cycler Dice Real Time System Lite and Thunderbird SYBR qPCR Mix, and actin was used as the reference gene in triplicate wells. Values are means with their standard errors represented by vertical bars. ■, CT; □, BL 0·5; ■, BL 5·0 (mg/ml). ** $P<0·01$, *** $P<0·005$ by Tukey's *t* test.

mgDf50 and *ok434* worms (Fig. 5(b) and (c)). These results suggested that heat stress tolerance and longevity was mediated by DAF-16 and SIR-2.1, respectively. This notion was supported by the finding that BL increased *hsp-12.6* expression (Fig. 7). Furthermore, BL induced the nuclear localisation of DAF-16 (Fig. 6(e)). These findings suggested that BL dose-dependently activated DAF-16. Previously, it was shown that DAF-16 nuclear translocation promotes longevity in *C. elegans*[18]. In addition, a previous study demonstrated that SIR-2.1 deacetylates and activates DAF-16[34]. Therefore, results of these and the present studies suggested that BL activates DAF-16 via SIR-2.1 to induce longevity.

In this study, the mRNA level of *sod-3*, a target gene of *daf-16*, did not increase, but it seems to be owing to the following reasons. The nuclear translocation of SMK-1, together with DAF-16, induces transcription of *sod-3*[35], whereas co-localisation of

DAF-16 with HSF-1 induces the gene expression of *hsp-12.6*[35,36]. In this study, HSF-1 could be activated and translocated in the nucleus with DAF-16. This notion was supported by the result that BL up-regulated the expression of *hsp-16.2* and *hsp-70*, the target genes of HSF-1. Therefore, the physiological effect of BL was due to DAF-16, but the contribution of these effects is thought to be *hsp-12.6* instead of *sod-3*.

This study also demonstrated that BL decreased fat accumulation (Fig. 6(a)) and increased mitochondrial membrane potential and ROS level (Fig. 6(b) and (c)) in worms. It is hypothesised that BL activated the mitochondria and oxidised fat in the process in *C. elegans*[37]. Typically, longer-living worms have lower mitochondrial ROS levels; however, a mild increase in ROS level was reported to induce mitohormesis and prolong life span[37,38].

Here, BL increased the recovery rate from heat stress (Fig. 3(d)) and H_2O_2 stress tolerance (Fig. 4(c)) in *tm4241* and wild-type N2 worms, suggesting that BL-induced heat and H_2O_2 stress tolerance was independent of SKN-1. However, BL failed to conserve the movement (Fig. 2(b)) and prolong life span (Fig. 5(d)) of *tm4241* worms. These results suggested that the longevity effect of BL was only partially mediated by SKN-1. Other studies have reported that other probiotics, *Bifidobacterium infantis* and *Lactobacillus gasseri* SBT2055, prolong the life span of worms via SKN-1, but is independent of DAF-16[39,40]. Surprisingly, results of this study showed that the anti-ageing effect of killed BL (BR-108) was partially mediated by SKN-1 and mainly via DAF-16. Although BL supplementation induced resistance to H_2O_2 stress in this study (Fig. 4(a) and (c)), the mechanism was unclear. Gene expression analysis showed increased *ctl-1* and *ctl-2* expression in BL-fed worms (Fig. 8). Catalases in worms may be activated to degrade H_2O_2; however, further studies are needed to elucidate the signalling pathway involved.

This study may be used as a model study to determine the effect of probiotics on ageing. Further studies evaluating the physiological effects of probiotics in higher animals are needed to better understand the benefit of killed probiotics.

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T. S. and K. S. conceived and designed the study, analysed data, wrote the paper and made manuscript revisions. T. S. performed all experiments. K. S. supervised the study as the principal investigator. All authors read and approved the final manuscript.

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Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114518001563

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