The Transcription Factor DAF-16 is Essential for Increased Longevity in *C. elegans* Exposed to *Bifidobacterium longum* BB68

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The longevity-promoting benefits of lactobacilli were hypothesized as early as 1907. Although the anti-aging effects of lactobacilli have been observed in nematodes, rodents and humans for over a century, the mechanisms underlying the effects of probiotics on aging have rarely been assessed. Using the *Caenorhabditis elegans* (*C. elegans*) model, various studies have elucidated the role of different signaling cascades, especially the DAF-16 cascade, on lifespan extension by LAB. In this study, the mechanisms through which *Bifidobacterium longum* strain BB68 affects the longevity of *C. elegans* were assessed. The lifespan of nematodes increased by 28% after worms were fed BB68, and this extension of lifespan was completely lost in backgrounds containing a mutated DAF-16 gene. High levels of DAF-16 (in the daf-16 (mu86); muIs61 strain) nuclear accumulation and high expression of the SOD-3 gene (a DAF-16-specific target gene) were observed as a result of BB68 treatment. Immunofluorescence microscopy revealed that TIR-1 and JNK-1 are involved in the phosphorylation and activation of DAF-16. Thus, BB68 increased the longevity of nematodes by activating the TIR-1 – JNK-1 – DAF-16 signaling pathway, and the cell wall component of BB68 contributed to longevity.

Lactobacilli were hypothesized to be vital bacteria for promoting human health and longevity as early as 1907. For a century, lactic acid bacteria (LAB), including bifidobacteria, have been commonly used as probiotic microorganisms due to their beneficial effects on the host. The anti-aging effects of these probiotics have been assessed in nematodes, rodents, and humans to evaluate improvements in immunosenescence in elderly animals. Using the *Caenorhabditis elegans* (*C. elegans*) model, several studies have described the mechanisms through which LAB affects aging (Table 1), including mechanisms involving different longevity signaling pathways related to stress resistance and calorie restriction. The DAF-2/DAF-16, p38 MAPK/SKN-1, and JNK-1/DAF-16 signaling pathways were found to contribute to stress resistance, which contributes to prolonged lifespan, and AAK-2/DAF-16 might be associated with calorie restriction-dependent longevity (Table 1). Two major transcription factors, SKN-1 and DAF-16, may act as key longevity regulators in worms that respond to various LAB strains. The species/strain-specific mechanisms through which probiotics affect the longevity of nematodes were identified in previous studies. In particular, the role of DAF-16 and its signal transduction were found to be inconsistent in different studies.

To study the anti-aging effects of probiotics, our laboratories isolated several LAB strains from centenarians living in an area with high longevity that may be responsible for the longevity of these individuals. Here, we show that *Bifidobacterium longum* BB68, a novel probiotic strain isolated from a centenarian living Bama, Guangxi, China.
Escherichia coli (E. coli) OP50 (Fig. 1A; Table S1). Moreover, the lifespan extension by BB68 fed standard food, BB68 to Bifidobacterium infantis also supported by a previous study, in which did not alter these indices 2. The bacterial SOD-3, one of the DAF-16-specific target genes, was determined by qPCR11. A significant increase in the expression to BB68 treatment. To confirm the role of DAF-16 in BB68-induced longevity, the level of gene expression of nuclear localization of DAF-16 protein (Fig. 1C). These results suggested the up-regulation of DAF-16 in response of DAF-16 by 56% (Fig. 1B; Table S3) relative to that induced by OP50. Western blotting also showed increased (mu86); muIs61), we demonstrated that BB68 significantly increased the nuclear accumulation strain (mu86)) caused the BB68-mediated effect of prolonged lifespan to be completely lost (Fig. 1A; Table S1). This phenomenon indicated that such an extension is DAF-16 dependent. Using a DAF-16-GFP fusion reporter daf-16 (e1368) mutants (Fig. 1A; Table S1; Fig. 1E), and activated JNK-1 was observed in the nerve ring of BB68-treated nematodes (Fig. 2). In addition, the BB68-induced nuclear translocation of DAF-16 was significantly increased in worm fed OP50. Western blotting also showed increased nuclear localization of DAF-16 protein (Fig. 1C). These results suggested the up-regulation of DAF-16 in response to BB68 treatment. To confirm the role of DAF-16 in BB68-induced longevity, the level of gene expression of SOD-3, one of the DAF-16-specific target genes, was determined by qPCR11. A significant increase in the expression of SOD-3 (2.27-fold compared to SOD-3 expression in worms fed OP50) was observed in worms fed BB68 for 24 h (Fig. 1D). Thus, we concluded that DAF-16 is essential for BB68-mediated lifespan prolongation.

DAF-2 is a human insulin receptor homolog upstream of DAF-16 in ILS, and mutations in DAF-2 decrease extensions in lifespan and resistance to pathogens by activating the nuclear translocation of DAF-16 16-18. Our results show that feeding BB68 to worms further prolongs the lifespan of daf-2 (e1368) mutants (Fig. 1A; Table S1), indicating that the BB68-mediated regulation of DAF-16 is independent of DAF-2.

In addition to functioning in ILS, DAF-16 is downstream of MAPK. To identify the regulator in the MAPK pathway involved in the BB68-induced, DAF-16-mediated regulation of the extension of lifespan, JNK and p38 were assessed. JNK-1 and PMK-1/p38, two members of the MAPK family in C. elegans, can transmit environmental stress signals to DAF-16 and regulate its nuclear localization 13, 14. Our results show that BB68 extends the lifespan of pmk-1 (km25) mutants, but not jnk-1 (gk7) mutants (Table S1; Fig. 1E), and activated JNK-1 was observed in the nerve ring of BB68-treated nematodes (Fig. 2). In addition, the BB68-induced nuclear translocation of DAF-16 was decreased in worms with a mutated JNK-1 gene (Fig. 1B; Table S3). Thus, JNK appears to be involved in the BB68-mediated regulation of DAF-16. Additionally, we found that the TIR-domain protein TIR-1 participates in the BB68-mediated longevity signaling cascade. BB68 did not increase the lifespan of tir-1 (ok1052) worms (Fig. 1E; Table S1), and mutations in TIR-1 suspended JNK-1 activation and BB68-induced DAF-16 nuclear accumulation (Table S3; Fig. 2). Therefore, we concluded that BB68 regulates DAF-16 through the TIR–JNK signal transduction pathway, an innate immunity signaling cascade conserved from nematodes to mammals.

To assess the potential active component of BB68 on nematode longevity, the cell wall component (CW) and cell wall-free extracts (CFE) of BB68 were separated and fed to nematodes. Compared to the CF or CFE of OP50, the CW of BB68 significantly increased the lifespan of C. elegans in a dose-response manner (P < 0.05); however,
the CFE of BB68 did not exert a similar effect (Fig. 3). Thus, these results indicate that the cell wall components of BB68 contribute to its effects on longevity in nematodes.

Our data confirm that *Bifidobacterium longum* strain BB68 prolongs the lifespan of *C. elegans* by regulating the activity of DAF-16, which is involved in a conserved immune signaling cascade (Fig. 4). Since these regulators are likely conserved across several species, the possibility of increasing the longevity of humans through the consumption of probiotics is elevated. Nevertheless, future studies are essential to determine the effect of *Bifidobacterium longum* BB68 on longevity in mammals.

**Methods**

**Bacterial strains and culture conditions.** *Bifidobacterium longum* strain BB68 was isolated from fecal samples of healthy centenarians in Bama County, Guangxi, China, and preserved in the laboratory. *E. coli* OP50 (OP50), a standard food for nematodes, was obtained from the *Caenorhabditis* Genetics Center (CGC, USA).

The BB68 strain was cultured at 37 °C in anaerobic conditions with GENbox anaer (bioMérieux, France) using MRS broth (LuQiao, China) for 18 h. OP50 was grown in LB broth (AoBoXing, China) at 37 °C for 18 h with agitation at 220 rpm. Bacteria were harvested by centrifugation at 4,000 × g for 15 min, washed twice with sterile M9 buffer, and centrifuged at 16,000 × g for 15 min at 4 °C to remove the supernatant. Then, the bacteria were adjusted to a final concentration of 0.4 mg/µL (wet weight) in M9 buffer; this slurry was used as concentrated bacteria and preserved at −80 °C. Before use, the concentrated bacteria were transferred into a 95 °C water bath for 30 min and used as heat-killed bacteria.

**Nematodes and growth conditions.** *C. elegans* Bristol strain N2, cat-2 (ad1116), daf-16 (mu86), daf-16 (mu86); mls61, daf-2 (e1368), pmk-1 (km25), jnk-1 (gk7), tir-1 (ok1052) were obtained from CGC. The strains jnk-1 (gk7); daf-16 (mu86); mls61 and tir-1 (ok1052); daf-16 (mu86); mls61 were constructed according to Michael Koelle’s *C. elegans* protocols (http://medicine.yale.edu/lab/koelle/protocols/index.aspx). The primers are listed below.

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**Figure 1.** *Bifidobacterium longum* BB68 extends the lifespan of *C. elegans* by regulating TIR-1 – JNK-1 – DAF-16 signaling. (A) Feeding worms BB68 increased the lifespan of the N2 and daf-2 strains, but not the daf-16 strain. (B) Feeding DAF-16::GFP worms BB68 for 24 h activated DAF-16 (the arrows indicate increased nuclear accumulation of DAF-16), and lower levels of activated DAF-16 were detected in the presence of mutated jnk-1 or tir-1. (C) Western blotting showed significant nuclear accumulation of DAF-16 protein in nematodes fed BB68 for 24 h. Lamin B1 was used as a nuclear reference, and GAPDH was used as a cytosolic reference. (D) An increase in SOD-3 gene expression level was observed in nematodes fed BB68 for 24 h. **Indicates a significant difference (Student’s T-test, p < 0.01). The test was conducted in triplicate. (E) The presence of mutant jnk-1 and tir-1 completely eliminated the lifespan extension induced by BB68. Scale bar = 50 μm.
**JNK1**

F: 5′-GTAGAAGCGTGGAAGAGGA-3′; JNK1 R: 5′-AGCTCTCCAAATATACACCC-3′. The wild-type amplicon is 1951 bp, and the jnk-1 (gk7) amplicon is 765 bp.

**TIR1**

F: 5′-AGATAAAGTCGGCAACCTGC; TIR1 R: 5′-CAAATGGCGATCTGTACCCT. The wild-type amplicon is 3224 bp, and the tir-1 (ok1052) amplicon is 1265 bp.

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**Figure 2.** Feeding N2 worms BB68 for 24 h activated JNK-1 via phosphorylation. The phospho-JNK/SAPK (Thr183/Tyr185) antibody and Cy3-labeled goat anti-mouse IgG (H + L) were used as primary and secondary antibodies, respectively. The dashed circle indicates phosphorylated JNK-1 in the nerve ring, but no activated JNK-1 was detected in the presence of mutant tir-1.

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**Figure 3.** The cell wall is the main effective component of BB68 on nematode longevity. (A) Feeding worms the cell wall (CW) of BB68 (0.12 mg and 1.2 mg per plate) significantly increased the lifespan of nematodes relative to feeding them OP50 CW (0.12 mg per plate). (B) Feeding worms cell wall-free extracts (CFE) of BB68 did not affect the lifespan of nematodes relative to the lifespan of nematodes fed OP50 CFE.

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JNK1 F: 5′-GTAGAAGCGTGGAAGAGGA-3′; JNK1 R: 5′-AGCTCTCCAAATATACACCC-3′. The wild-type amplicon is 1951 bp, and the jnk-1 (gk7) amplicon is 765 bp.

TIR1 F: 5′-AGATAAAGTCGGCAACCTGC; TIR1 R: 5′-CAAATGGCGATCTGTACCCT. The wild-type amplicon is 3224 bp, and the tir-1 (ok1052) amplicon is 1265 bp.
All *C. elegans* strains were maintained at 25 °C (unless otherwise stated) on nematode growth medium (NGM) agar supplemented with OP50 using standard techniques. For the lifespan assay, mNGM plates were produced by supplementing the NGM plates with filter-sterilized carbenicillin (0.5 mM) and 5-fluoro-2′-deoxyuridine (FUdR) (50 μM). Heat-killed bacteria (10 mg) were spread on 60 mm mNGM plates.

**Lifespan assay.** L4 stage nematodes grown on NGM plates were transferred to mNGM plates with a platinum wire. For each lifespan assay, 100 worms for every bacterial species were assayed in ten plates (ten worms/plate), and plates were cultured at 25 °C. The number of live and dead worms was determined using a dissecting microscope (Chong Qing Optical, China) every 24 h. The lifespan assay was conducted at least three times. The nematode survival rate was calculated using the Kaplan–Meier method, and differences in survival rates were evaluated for significance using the log-rank test (*P* < 0.05).

**Food gradient feeding assay.** Heat-killed BB68 and OP50 bacterial suspensions were spread on mNGM plates in serial concentrations ranging from 0.01–200 mg bacterial cells/plate. L4 stage N2 worms were placed on these plates. For each concentration, 100 worms were assayed over ten plates (ten worms/plate). Lifespan was measured as described previously. The test was conducted at least three times.

**Measurements of body size, pharynx pumping rate, and reproduction.** L4 larvae were placed on NGM plates coated with bacterial lawns. Ten worms/bacterial species were assayed using ten plates (one worm/plate). Beginning on the first day that *C. elegans* were transferred to fresh NGM plates, the size of the live worms was examined every 24 h. Images of adult nematodes were captured using an XSP-8CZ digital microscope (Chong Qing Optical), and the projection area of the worms was analyzed as the body size using ImageJ software (National Institutes of Health, USA). To determine the pharynx pumping rate, L4 nematodes were grown on NGM plates in the presence of bacterial lawns for 30 min before counting. The pumping frequency was recorded as the number of contractions in the terminal bulb of the pharynx of an individual worm in a 60-s period. The experiment was performed five times. For the reproduction assay, the worms were transferred daily to fresh NGM plates until reproduction ceased. The offspring of each animal were counted at the L2 or L3 stage. The test was repeated three times.

**Determination of SOD-3 gene expression.** Total RNA was extracted from L4 stage worms using TRNzol (Tiangen, China) accompanied by BeadBeater-16 (BioSpec, USA). Reverse transcription was completed with a Quantscript RT Kit (Tiangen). The expression of the SOD-3 gene was detected using the primers SOD3F: 5′-GGTTGCGGGAGTTCTCGCCG-3′ and SOD3R: 5′-TCCCTTTCGAAACAGCCTCGG-3′ on a Quantica Real-Time PCR system (Techne, UK). The ACTIN-2 gene was used as an internal reference gene.

**DAF-16::GFP localization assay.** L4 larvae of DAF-16::GFP worms were scored for nuclear, cytoplasmic, or intermediate fluorescent protein (GFP) localization as described by Oh *et al.* The number of worms with each level of nuclear translocation was determined. The percentage of worms showing nuclear and intermediate fluorescent translocation out of 100 worms was calculated as the DAF-16 nuclear location %. The assay was conducted five times.

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**Figure 4.** Mechanism by which BB68 affects the longevity of *C. elegans*. BB68 increased the longevity of nematodes by activating the TIR-1 – JNK-1 – DAF-16 signaling pathway, and the cell wall component of BB68 contributed to longevity. The solid arrow indicates activation observed in this study. A dashed line with an ‘×’ indicates that no effect was observed in this study. A dashed line indicates that the test was not performed in this study. A “P” indicates a phosphorylation site. *Indicates direct or indirect phosphorylation.
Nuclear and cytosolic distribution of DAF-16 (Western blotting). The L4 larvae of DAF-16::GFP worms were collected. Subsequently, the nuclear and cytosolic fractions were separated, and the DAF-16 protein was detected by Western blotting as described by Singh and Aballay. Lamin B1 and GAPDH were used as markers for the nuclear and cytosolic fractions, respectively. Antibodies against GAPDH, DAF-16, and lamin B1 were obtained from Cell Signaling Technology (CST, USA), Santa Cruz Biotechnology (USA), and Abcam (USA), respectively.

Immunofluorescence staining assay. Immunofluorescence staining for phosphorylated-JNK-1 in L4 larvae was carried out according to Michael Koelle’s C. elegans protocols (http://medicine.yale.edu/lab/koelle/protocols/index.aspx). A phospho-JNK/SAPK (Thr183/Tyr185) antibody and Cy3-labeled goat anti-mouse IgG (H + L) (Beyotime Biotech, China) were utilized as primary and secondary antibodies, respectively. Fluorescence images were obtained with a LEICA Axioscope microscope with excitation at 550 nm.

Separation of CW and CFE of bacteria. OP50 and BB68 were cultured, and cells were harvested by centrifugation at 14,000 × g for 10 min at 4°C. Then, the CW and CFE were separated by sonication as described by Komura et al. The CW and CFE were freeze dried and stored at −80°C until further use. For the lifespan assay, CW and CFE were solubilized at 50 mg/mL in M9 buffer and added to heat-killed OP50 in a concentration gradient. Fifty microliters of the mixture was spread on 35 mm mNGM plates containing 3.4 mg of heat-killed OP50 and different concentrations of CW and CFE (0.012 mg, 0.12 mg, and 1.2 mg as low, medium, and high concentrations, respectively). Then, the lifespan assays were performed as described above. The medium concentration (0.12 mg) of CW or CFE from OP50 was used as a control in the lifespan assays.

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Author Contributions
L. Zhao designed and performed experiments, and prepared manuscript. Y. Zhao performed the lifespan assays. R. Liu revised the manuscript and improved English writing. X. Zheng performed separation of C.W. and C.F.E. of bacteria. M. Zhang and H. Zhang helped with immunofluorescence staining assay and western blotting. H. Guo revised the manuscript and helped with DAF-16 determination. F. Ren designed the experiments, and revised manuscript.

Additional Information
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