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Full Paper

Bacillus subtilis TO-A extends the lifespan of *Caenorhabditis elegans*

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***Clostridium butyricum* TO-A, *Enterococcus faecium* T-110, and *Bacillus subtilis* TO-A are sold as oral probiotic preparations and reportedly exhibit many beneficial effects on the health of hosts, including humans and livestock. In this study, we compared the ability of these clinically applied probiotic bacteria with *Escherichia coli* OP50 in extending the lifespan of *Caenorhabditis elegans*. To compare the *C. elegans* lifespan-extending effects of the three bacteria, experiments were performed using a nematode growth medium containing a small amount of trypticase soy agar. The maximum lifespans of worms fed *C. butyricum* TO-A, *E. faecium* T-110, or *B. subtilis* TO-A increased by 11, 12, and 26%, respectively, compared with worms fed *E. coli* OP50. In addition, we conducted a metabolomic analysis of methanol extracts of *B. subtilis* TO-A cells, which exhibited the strongest lifespan-extending effect on *C. elegans* among the probiotic bacteria tested in this study. As a result, 59 candidate substances involved in extending the lifespan of *C. elegans* were identified in *B. subtilis* TO-A cells.**

Key words: *Clostridium butyricum*, *Enterococcus faecium*, *Bacillus subtilis*, *Caenorhabditis elegans*, lifespan

INTRODUCTION

Many studies have reported that probiotics are effective in treating a number of diseases, including acute infectious diarrhea, antibiotic-associated diarrhea, *Clostridioides difficile*-associated diarrhea, hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, functional gastrointestinal disorders, and necrotizing enterocolitis, and high-quality evidence suggests they are safe in a wide range of populations, including infants, children, adults, and older patients [1]. Probiotics help maintain host health by regulating the balance and homeostasis of the intestinal microbiota [1–3]. Oral probiotic preparations that contain *Clostridium butyricum* TO-A (CB), *Enterococcus faecium* T-110 (EF), and *Bacillus subtilis* TO-A (BS) exert beneficial effects on the health of hosts, including humans and livestock. Preparations containing these organisms have been utilized as probiotic drugs for treating intestinal diseases such as constipation, diarrhea, irritable bowel syndrome, enteritis, and other infectious diseases for more than 50 years [4–8]. In addition, it was recently suggested that these probiotic bacteria could reduce the risk of recurrent spontaneous preterm delivery in pregnant women [9]. However, there are still many unresolved questions regarding how interactions between these bacteria impact host health. We hypothesized that the beneficial effects of these bacterial strains on host health can be assessed by observing their effects in extending the lifespan of the host. This is because the extension of lifespan derives from improvement of quality of life, facilitation of disease prevention, and suppression of aging.

We focused on *Caenorhabditis elegans* as a model animal to elucidate the mechanisms of interactions between the bacteria and their host. As *C. elegans* is a bacterivore, it is usually cultured and passaged on lawns of *Escherichia coli* OP50 (hereinafter referred to as OP50). Hence, the intestinal bacteria of *C. elegans* could be defined as being composed of only a single strain of OP50. Moreover, the intestines of worms have very simple structures compared with other model animals, such as mice and flies [10], and the eggs of worms are resistant to exposure to hypochlorite, permitting the sterilization of eggs to prevent contamination with other bacteria, yeasts, and fungi [11]. Thus, if OP50 is exchanged with another bacterial strain as the feed for *C. elegans*, the effect of a single bacterial strain can be investigated more easily in *C. elegans* than in other model animals.

In the present study, we developed a new nematode growth medium (NGM), designated NGM-T, in which the standard NGM was amended with a small amount of trypticase soy agar (TSA) for culturing high nutrient-demand bacteria such as CB and other strains. Additionally, we investigated the lifespan-extending effect of various clinically employed probiotic bacteria, including, CB, EF, and BS, on worms. Finally, we conducted a metabolomic analysis of methanol extracts from BS cells, which

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exhibited the strongest lifespan-extending effect on *C. elegans*, in comparison with that of OP50.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In this study, we used the following bacteria: OP50, *B. subtilis* 168, CB, EF, and BS. OP50 was grown overnight in 5 mL of Luria-Bertani (LB) broth with shaking at 37°C. The broth was formulated using 1 g tryptone (Bacto, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 0.5 g yeast extract (Bacto, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 0.5 g NaCl, and 100 mL H_2O (pH 7.0). CB was grown for 4 hr in 5 mL of Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical, Tokyo, Japan) broth without shaking at 37°C under anaerobic conditions. EF was grown overnight in 5 mL of *Lactobacillus* medium based on the formulation of deMan, Rogosa and Sharpe (MRS, Difco, BD, Franklin Lakes, NJ, USA) broth without shaking at 37°C. BS and *B. subtilis* 168 were grown overnight in 5 mL of trypticase soy (TS) broth, created by removing agar from TSA (Eiken, Tokyo, Japan), with shaking, at 37°C. Following initial growth, cultures were adjusted to an optical density at 600 nm (OD600) of 0.5 using the relevant medium (LB, GAM, MRS, or TS broth). Then, 100 µL of bacterial suspension was spotted onto a 60-mm NGM-T (NGM amended with TSA) plate. The plate contained 2.69 g NaCl, 16.1 g agar, 2.5 g peptone (Bacto, Gibco, Thermo Fisher Scientific), 2.5 g TSA, 25 mL 1 mol·L-1 KPO4 buffer (108.3 g of KH_2PO_4, 35.6 g K_2HPO_4, 1 L H_2O), 1 mL 1 mol·L^{-1} $MgSO_4$, 1 mL 1 mol·L^{-1} CaCl2, 1 mL 5 mg·mL^{-1} cholesterol (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) in ethanol, and 975 mL H_2O. Subsequently, OP50, *B. subtilis* 168, EF and BS plates were incubated for 24 hr at 37°C under aerobic conditions, and CB plates were incubated for 24 hr at 37°C under anaerobic conditions.

Caenorhabditis elegans growth conditions

C. elegans N2 Bristol was used in this study and maintained at 20°C on NGM plates. The plates contained 3 g NaCl, 17 g of agar, 2.5 g peptone (Bacto, Gibco, Thermo Fisher Scientific), 25 mL 1 mol·L^{-1} KPO_4 buffer, 1 mL 1 mol·L^{-1} $MgSO_4$, 1 mL 1 mol·L^{-1} $CaCl_2$, 1 mL 5 mg·mL^{-1} cholesterol (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) in ethanol, and 975 mL H_2O and were seeded with OP50. The *C. elegans* strain was provided by the Caenorhabditis Genetics Center (CGC). For synchronization of the worms, 10 adult hermaphrodites were transferred to fresh NGM plates seeded with OP50 and cultured at 20°C for 6 hr. After the adult worms were removed, eggs that had been deposited onto the NGM plate were grown to adulthood at 20°C [12].

Bacterial lysate

Bacteria cultured on 60-mm NGM-T plates (see *Bacterial strains and growth conditions*) were transferred to 15-mL tubes using a spatula, and M9 buffer (3.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 5.0 g NaCl, 20 mg gelatin, 1 mL 1 M $MgSO_4$, and 1 L H_2O) was added at a ratio of 1 mL per 5 plates of bacteria. Subsequently, one-third of the final volume of Φ0.3-mm zirconia beads was added to each 15-mL tube, and the bacterial suspension was frozen at 80°C. After thawing, the bacteria were crushed at 6.5 m sec^{-1} for 50 sec using a FastPrep-24 system (MP Biomedicals, Santa Ana, CA, USA) and left standing on ice to cool for 2 min (this process was repeated 7 times). The bacterial lysate was then centrifuged at 2,000 × g for 5 min, and the supernatant was recovered and filtered through a 0.45-µm PVDF membrane filter (Puradisc 25, Whatman, Cytiva, Marlborough, MA, USA). Finally, 200 µL of the supernatant was spotted onto a lawn of OP50 cultured on a 60-mm NGM-T plate.

Lifespan assay

The lifespan assay was modified from a previously reported method [13]. Synchronized worms that had been grown to adulthood were collected with M9 buffer and washed three times. Subsequently, 10–20 washed worms were transferred to the center of a bacterial lawn. Worms were monitored at 20°C and transferred to fresh (bacteria-inoculated) plates every other day. A worm was considered dead if it did not exhibit pharyngeal pumping and did not react to prodding with a picker. Worms that died as a result of internal hatching or as a result of climbing the plate wall were removed from the plate and not included in the assay. All experiments were performed three times.

Bacterial cell extraction

Extracts of BS and OP50 cells were prepared as follows. Bacteria grown on NGM-T for 24 hr at 37°C were collected in Milli-Q water, and the OD_{600} was determined as shown in Supplementary Table 1. The collected bacteria were trapped by passage through a 0.4-µm Isopore membrane filter (Millipore, Merck KGaA, Darmstadt, Germany), and the filter was then washed twice with 10 mL of Milli-Q water. The membrane filter was immersed in 1.6 mL of methanol in a petri dish, after which the trapped bacteria were dissociated from the filter by ultrasonication for 30 sec. A 1.1-mL volume of internal standards (H3304-1002, Human Metabolome Technologies, Inc. [HMT], Tsuruoka, Yamagata, Japan) diluted in Milli-Q water was added to the bacterial cell extract, and the mixture was allowed to stand for 30 sec at room temperature and then immediately cooled on ice. Following centrifugation at 4°C and 2,300 × g for 5 min, 350 µL of the resulting supernatant was placed on a Millipore 5-kDa cutoff filter (UltrafreeMC-PLHCC, HMT), and the filter was centrifuged at 4°C and 9,100 × g for 3.5 hr. At HMT, the resulting filtrate was lyophilized, and the residue was resuspended in 25 µL of Milli-Q water and subjected to analysis of bacterial metabolites.

Metabolomic analysis

The metabolomic analysis was performed by HMT using capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) [14, 15]. The bacterial extract was prepared as follows: the sampling volume (mL) was 20 per optical density at 600 nm, and the results were compared with data in the HMT Basic Scan package. The equipment and assay conditions used for the metabolomic analysis are shown in Supplementary Table 2. Using automatic integration software (MasterHands, Keio University, Tsuruoka, Yamagata, Japan), each peak with a signal-to-noise ratio >3 was extracted from the detected CE-TOFMS peaks to permit acquisition of the m·z^{-1} value, peak area value, and migration time (MT) [16]. Detected peaks related to adduct ions, isotopomers, and other product ions of known metabolites were excluded. Based on their m·z^{-1} and MT values, the remaining peaks were collated against all substances in the HMT metabolite

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library. To obtain the relative area of each metabolite, the detected peak area was corrected according to the peak area of the internal standard and sample volume (OD_{600} mL^{-1}). Principal component analysis (PCA) was performed using HMT's proprietary statistical analysis software [17].

Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics (Ver 23.0.0.0, IBM Corp., Armonk, NY, USA). The lifespan assay data were evaluated by log-rank test, whereas other data were evaluated by Welch's t-test. A p-value <0.05 was defined as statistically significant. Where indicated, values are annotated as ns (not significant; p>0.05), *p<0.05, **p<0.01, or ***p<0.001.

RESULTS

Characteristics of the newly developed medium

NGM is commonly used for experiments involving *C. elegans*. However, NGM is nutrient poor, which makes it difficult to culture bacteria that require high amounts of nutrients for proliferation. Indeed, CB did not form a lawn on NGM (Supplementary Fig. 1a). Therefore, we developed a medium consisting of NGM amended with TSA, and the resulting medium, NGM-T, was sufficient for CB lawn formation (Supplementary Fig. 1a). TSA is a versatile medium with a simple composition that does not contain inhibitors or indicators, and it has been used in many previous studies [18]. However, OP50 cultured on TSA reportedly decreases the lifespan of worms [19]. We obtained similar results, in that the lifespan of worms fed OP50 cultured on TSA was decreased (Supplementary Fig. 1b). The lifespan of worms fed OP50 cultured on NGM-T was slightly shorter than the lifespan of worms fed OP50 cultured on NGM (Supplementary Fig. 1c). In addition, other characteristics of the worms, such as growth rate, body length, number of eggs produced, and defecation cycles, were similar between worms fed OP50 cultured on NGM-T and worms fed OP50 cultured on NGM (Supplementary Fig. 2). Based on these results, we concluded that the properties of NGM-T are similar to those of NGM and better than those of TSA and that NGM-T is suitable for use in *C. elegans* experiments.

Comparison of the C. elegans *lifespan-extending effects of three probiotic bacterial strains*

Previous studies reported that compared with OP50, strains of *C. butyricum*, *E. faecium*, and *B. subtilis* extend the lifespan of worms [13, 20–22]. In fact, we confirmed that worms fed *B. subtilis* 168 cultured on NGM-T exhibited extended lifespans (Fig. 1a). A previous report indicated that *B. subtilis* 168 cultured on NGM exerts a lifespan-extending effect [20]. Therefore, the clinically employed probiotic bacteria CB, EF, and BS were also expected to exhibit lifespan-extending effects in worms. Indeed, our results showed that the maximum lifespans of worms fed CB, EF, or BS increased by 11%, 12%, and 26%, respectively, compared with the worms fed OP50 (Fig. 1 and Table 1). In particular, the lifespans of worms fed BS increased by 6 days compared with worms fed OP50, which was much longer than the lifespans of worms fed the other two strains of probiotic bacteria (Fig. 1d and Table 1). Additionally, the worm lifespan-extending effect of BS was shown to be more potent than that of the *B. subtilis* 168 strain of the same species (Table 1). Furthermore, lifespan extension was also observed in *C. elegans* fed BS cultured on NGM (Supplementary Fig. 3). These data suggest that the lifespan-extending effect of BS on *C. elegans* was not markedly affected by the culture medium.

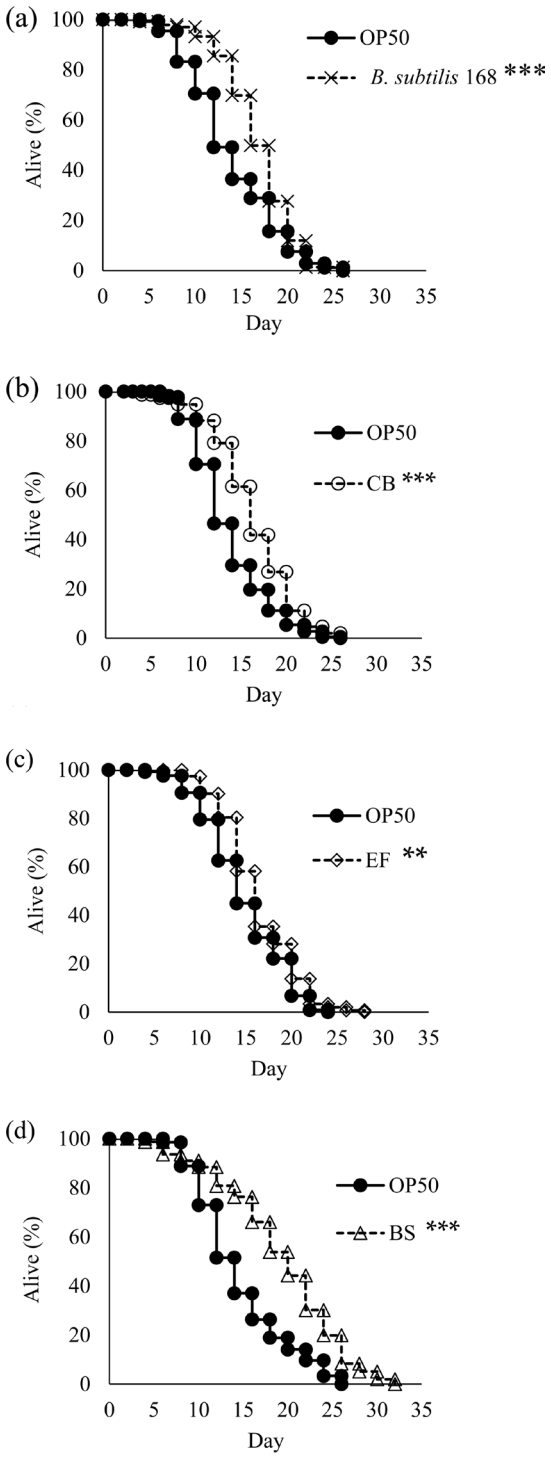

Fig. 1. Worm lifespan-extending effects of probiotic bacteria. Survival curves represent data for worms fed (a) *B. subtilis* 168, (b) *Clostridium butyricum* TO-A (CB), (c) *Enterococcus faecium* T-110 (EF), or (d) *Bacillus subtilis* TO-A (BS). Survival curves indicate the mean of three independent experiments: (a) n=173 for OP50, n=235 for *B. subtilis* 168; (b) n=224 for OP50, n=153 for CB; (c) n=254 for OP50, n=153 for EF; (d) n=270 for OP50, n=156 for BS. **p<0.01 and ***p<0.001 as determined by log-rank test.

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Table 1. Effect of probiotic bacteria on worm lifespan

Bacteria		Mean lifespan			Maximum lifespan	
		Mean ± S.E. (Day)		Rate of increase (%)	Mean ± S.E. (Day)	Rate of increase (%)
B. subtilis 168 group	OP50	13.8 ± 0.36		-	23.3 ± 1.1	-
	B. subtilis 168	16.7 ± 0.25	***	21	26.0 ± 0	11
CB group	OP50	13.5 ± 0.27		-	23.3 ± 1.4	-
	CB	16.1 ± 0.35	***	20	26.0 ± 0	11
EF group	OP50	14.7 ± 0.27		-	22.7 ± 1.1	-
	EF	16.2 ± 0.33	**	10	25.3 ± 1.1	12
BS group	OP50	14.4 ± 0.30		-	23.3 ± 1.4	-
	BS	19.3 ± 0.52	***	34	29.3 ± 1.4	26

The table was created based on the data shown in Fig. 2. CB: *Clostridium butyricum* TO-A; EF: *Enterococcus faecium* T-110; BS: *Bacillus subtilis* TO-A; S.E.: standard error of the mean. **p<0.01 and ***p<0.001 as determined by log-rank test.

Investigation of C. elegans *lifespan-extending compounds from extracts of BS cells*

We sought to identify bacterial metabolites involved in the lifespan-extending effect on worms. Specifically, we compared the compounds isolated from extracts of OP50 and BS cells, which showed the strongest lifespan-extending effect on worms among the probiotic bacteria tested in our study. The lifespan-extending effects on worms of other strains of *B. subtilis* have been reported [13, 23–25]. Moreover, the release of competence- and sporulation-stimulating factor (also called the quorum-sensing pentapeptide) and nitric oxide by *B. subtilis* is reportedly involved in extending worm lifespan [13]. The difference in lifespan between *B. subtilis*-fed and OP50-fed worms has been attributed to the supply of dietary coenzyme Q obtained by worms fed OP50 [23]. However, to our knowledge, no previous report has described a comprehensive search for compounds from extracts of *B. subtilis* that affect the lifespan of worms. Bacteria ingested by worms are crushed in the pharynx and transported to the intestine, where various nutrients are absorbed. Therefore, we hypothesized that the extracts of BS cells would contain many substances potentially involved in lifespan extension in worms. Indeed, we showed that the addition of BS lysate to OP50 plates extended the lifespan of worms (Fig. 2a). However, the addition of OP50 lysate to OP50 plates did not extend the lifespan of worms (Fig. 2a). The results shown in Fig. 2a suggest that components in extracts of BS cells are involved in lifespan extension in worms and that the worm lifespan-extending effect of BS is not due to caloric restriction. In the metabolomic analysis, 410 compounds were detected in CE-TOFMS in either the cation or anion mode (Supplementary Table 3). PCA of these metabolites showed that the metabolomic profile of BS cells differed markedly from that of OP50 cells (Fig. 2b). A total of 175 compounds showed significant differences in abundance between BS and OP50 cells, and the amounts of 59 compounds were higher in BS than in OP50 (Fig. 2c, Table 2 and Supplementary Table 3). Moreover, 11 of these compounds were previously reported as being involved in lifespan extension in worms (Supplementary Table 4) [26–29]. Although the mechanistic relationship between metabolites and lifespan extension in worms has not been elucidated, citric acid (involved in protection from heavy metal stress), gamma-aminobutyric acid (involved in protection from neurodegeneration), and butyric acid (involved in inhibition of

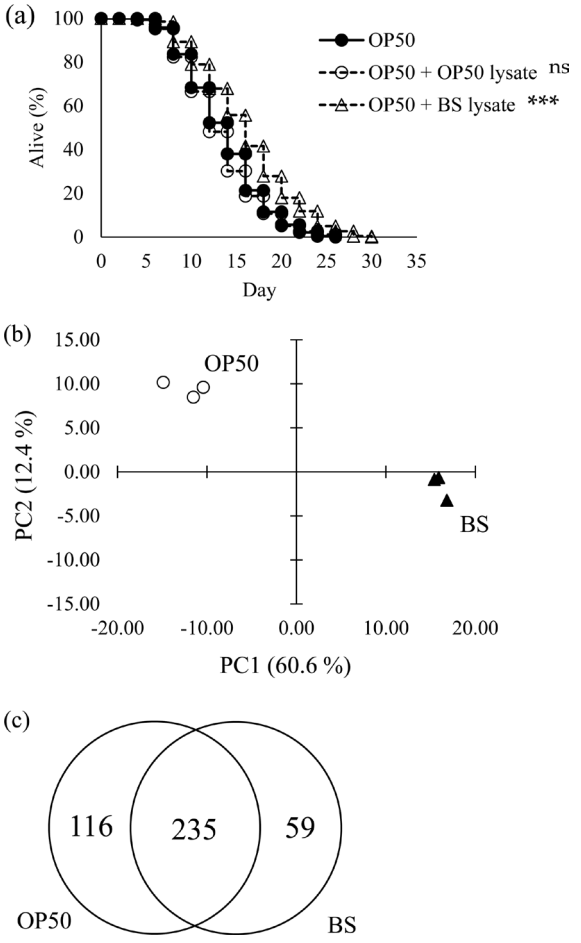

Fig. 2. Metabolomic analysis of components from extracts of *Bacillus subtilis* TO-A (BS) cells compared with those of OP50 cells. (a) Survival curves represent data for worms fed OP50 with the addition of OP50 lysate or BS lysate or OP50 without addition of anything (n=287 for OP50, n=272 for OP50 + OP50 lysate, n=264 for OP50 + BS lysate). Ns (not significant; p>0.05) and ***p<0.001 as determined by log-rank test. In log-rank tests, OP50 was used as the control group. (b) Metabolomic profiles of BS cells and OP50 cells as visualized by principal component analysis (PCA). (c) Venn diagram showing the number of compounds that were significantly enriched in BS cells compared with OP50 cells. The overlap region indicates the number of compounds that did not show a significant difference in abundance between BS cells and OP50 cells.

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Table 2. Compounds more abundant in extracts of BS cells compared with OP50 cells

Compound name	Relative area (Mean ± S.E.)		BS vs. OP50		
	OP50	BS	Ratio¶	p-value‖	
2,6-Diaminopimelic acid	1.40E-03 ± 5.50E-05	7.30E-02 ± 3.1.E-03	52	0.003	**
2-Aminoisobutyric acid 2-Aminobutyric acid	5.30E-04 ± 1.40E-05	2.80E-03 ± 1.6.E-04	5.3	0.007	**
2-Hydroxyglutaric acid	5.10E-05 ± 4.60E-06	1.10E-04 ± 2.8.E-06	2.2	0.046	*
3-Amino-2-piperidone	2.00E-04 ± 2.50E-05	3.50E-04 ± 6.9.E-06	1.8	0.031	*
5-Hydroxylysine	1.30E-03 ± 8.20E-05	5.00E-03 ± 1.1.E-04	3.7	<0.001	***
Acetoacetamide	1.50E-03 ± 1.70E-04	2.40E-03 ± 9.9.E-05	1.6	0.03	*
Alanine	4.50E-03 ± 4.60E-04	2.40E-02 ± 1.4.E-03	5.3	0.004	**
Argininosuccinic acid	7.40E-05 ± 3.20E-06	2.30E-04 ± 1.6.E-06	3.2	<0.001	***
Asparagine	1.30E-02 ± 3.00E-03	2.30E-02 ± 9.7.E-05	1.8	0.013	*
Betaine	4.20E-03 ± 6.30E-04	3.80E-02 ± 2.0.E-03	9	0.003	**
Cholic acid	7.40E-05 ± 3.40E-06	4.40E-04 ± 2.9.E-05	6	0.008	**
Citric acid	1.00E-03 ± 7.30E-05	2.40E-02 ± 5.0.E-04	23	<0.001	***
Citrulline	5.10E-04 ± 8.30E-05	6.90E-03 ± 2.5.E-04	13	<0.001	***
Dyphylline	1.50E-04 ± 1.50E-05	5.60E-04 ± 2.2.E-05	3.8	<0.001	***
Fructose 1,6-diphosphate	1.40E-04 ± 2.90E-05	5.30E-04 ± 2.0.E-05	3.7	0.002	**
Fructose 6-phosphate	1.10E-04 ± 1.40E-05	4.00E-04 ± 2.8.E-05	3.8	0.005	**
γ-aminobutyric acid	8.30E-04 ± 7.70E-05	7.30E-03 ± 7.7.E-04	8.7	0.02	*
Glutamine	3.50E-02 ± 4.30E-03	5.40E-02 ± 3.2.E-03	1.5	0.049	*
Glutamic acid	4.30E-03 ± 2.20E-04	2.20E-02 ± 9.3.E-04	5.1	0.003	**
Glucose 6-phosphate	2.50E-04 ± 3.10E-05	1.50E-03 ± 7.8.E-05	5.9	0.002	**
Glycine	1.60E-03 ± 1.60E-04	1.70E-02 ± 9.0.E-04	11	0.004	**
Glycerophosphocholine	4.30E-04 ± 3.80E-05	8.60E-04 ± 2.1.E-05	2	0.004	**
Glycocholic acid	2.10E-04 ± 1.90E-05	6.90E-04 ± 4.7.E-05	3.3	0.007	**
Guanine	1.80E-04 ± 3.50E-05	8.60E-04 ± 5.5.E-05	4.7	0.002	**
Homocitrulline	1.70E-04 ± 2.60E-06	1.30E-03 ± 3.6.E-05	7.7	0.001	**
Hydroxyproline	8.10E-03 ± 8.00E-04	2.30E-02 ± 1.3.E-03	2.8	0.003	**
Isobutyric acid Butyric acid	3.70E-04 ± 3.60E-05	4.80E-03 ± 4.0.E-04	13	0.011	*
Isovaleric acid DL-2-Methylbutyric acid Valeric acid	2.10E-04 ± 5.40E-06	5.70E-04 ± 3.0.E-05	2.8	0.008	**
Lactic acid	2.60E-03 ± 8.60E-05	4.30E-03 ± 1.8.E-04	1.7	0.007	**
Methionine	3.00E-02 ± 1.30E-03	4.50E-02 ± 2.2.E-03	1.5	0.014	*
N-Acetylaspartic acid	3.20E-04 ± 2.50E-05	2.80E-03 ± 1.1.E-04	8.6	0.002	**
N-Acetyllysine	1.00E-02 ± 5.40E-04	3.90E-02 ± 8.2.E-04	3.7	<0.001	***
N^6-Acetyllysine	2.50E-04 ± 3.90E-05	5.40E-03 ± 8.6.E-05	21	<0.001	***
Ornithine	9.60E-04 ± 2.00E-05	3.30E-03 ± 8.5.E-05	3.4	0.001	**
Pipecolic acid	1.10E-03 ± 1.70E-04	9.60E-03 ± 5.8.E-04	8.6	0.004	**
Proline	6.90E-03 ± 4.00E-04	2.30E-02 ± 2.1.E-03	3.3	0.022	*
Serine	1.10E-03 ± 4.30E-05	8.90E-03 ± 2.3.E-04	8.3	<0.001	***
Stachydrine	2.10E-04 ± 4.10E-05	1.90E-03 ± 6.7.E-05	9.4	<0.001	***
Taurocholic acid	2.10E-04 ± 1.00E-05	8.30E-04 ± 5.8.E-05	3.9	0.011	*
Threonine	9.70E-04 ± 6.60E-05	1.10E-02 ± 1.2.E-04	11	<0.001	***
Trimethylamine	2.10E-04 ± 3.40E-05	1.20E-03 ± 8.2.E-05	5.8	0.004	**
Tryptophan	5.30E-04 ± 6.90E-06	6.40E-03 ± 2.0.E-04	12	0.002	**
UDP-glucuronic acid	2.00E-03 ± 9.80E-05	2.60E-03 ± 8.8.E-05	1.3	0.028	*
Urea	8.70E-03 ± 8.00E-04	3.40E-02 ± 1.4.E-03	3.8	<0.001	***
Urocanic acid	9.70E-05 ± 9.30E-06	4.20E-04 ± 3.9.E-05	4.3	0.017	*
XA0033	2.70E-04 ± 3.70E-05	1.30E-03 ± 4.0.E-05	4.8	<0.001	***
Xanthine	3.50E-04 ± 2.40E-05	1.90E-03 ± 9.8.E-05	5.3	0.004	**
XC0017	1.40E-04 ± 2.30E-06	5.20E-04 ± 3.0.E-05	3.8	0.009	**
XC0132	7.00E-05 ± 2.10E-06	3.40E-03 ± 7.9.E-05	49	<0.001	***
β-Alanine	9.60E-05 ± 2.10E-06	1.60E-04 ± 3.5.E-06	1.6	<0.001	***
β-Hydroxyisovaleric acid	8.00E-05 ± 7.40E-06	6.70E-04 ± 3.2.E-05	8.4	0.003	**
γ-Butyrobetaine	7.30E-04 ± 7.90E-05	6.00E-03 ± 2.5.E-04	8.2	0.002	**
γ-Glutamic acid-Aspartic acid	1.80E-04 ± 2.80E-05	4.40E-03 ± 2.1.E-04	24	0.003	**
γ-Glutamic acid-Ornitine	1.20E-04 ± 7.60E-06	3.50E-03 ± 1.6.E-04	29	0.003	**
γ-Glutamic acid-Serine	2.90E-04 ± 3.80E-05	1.80E-03 ± 4.0.E-05	6	<0.001	***

BS: *Bacillus subtilis* TO-A. The table was created based on the data shown in Supplementary Table 1. ¶Ratios of mean values between the two groups were calculated with the value for OP50 cells as the denominator. Glu was used to determine the relative area of ^{13}C because the intensity of the metabolite's peaks was saturated. S.E.: standard error of the mean.

protein aggregation by bacteria) were included among the 59 aforementioned compounds (Table 2) [30–32]. These data suggest that at least some of the 59 compounds that were significantly enriched in BS cells compared with OP50 cells play a role in extending the lifespan of *C. elegans*.

DISCUSSION

Using NGM-T, our study revealed that three clinically employed probiotic bacteria, CB, EF, and BS extend the lifespan of *C. elegans* and that BS exhibited the strongest lifespan-extending effect among these probiotic bacteria (Fig. 1 and Table 1).

In this study, we did not employ a method of applying pelleted bacteria, cultured on a medium other than NGM, to NGM. This is because the amounts and types of compounds produced by bacteria are strongly affected by the medium on which they are cultured. When pelleted bacteria cultured on a medium other than NGM are applied to NGM, the reproducibility of experimental results cannot be guaranteed, as the amounts and types of metabolites produced by the bacteria may change during the experiment. NGM is generally used as the standard medium for *C. elegans* experiments to fulfill the purpose of suppressing the growth of OP50 and making it easy to observe the worms on the OP50 lawn [33]. Hence, NGM is not suitable for culturing bacteria with high nutrient demands, and indeed, CB did not form a lawn on NGM in our experiments (Supplementary Fig. 1a). For this reason, we developed a new medium named NGM-T for culturing CB, which has a high nutrient demand. This medium was also used for comparing the clinically employed probiotic bacteria CB, EF, and BS with OP50 in terms of their effects on *C. elegans* lifespan. NGM-T exhibited fewer adverse effects on the lifespan of worms than TSA, which is often used for bacterial culture, and NGM-T contains more nutrients necessary for bacterial growth than NGM (Supplementary Fig. 1b and Supplementary Fig. 1c). Therefore, we concluded that NGM-T, like NGM, can be used to investigate the effects of the various bacteria on the lifespan of *C. elegans*.

Our metabolomic analysis identified 59 candidate metabolites that may contribute to the extension of the lifespan of *C. elegans*. These compounds were enriched in BS cells compared with OP50 cells (Fig. 2 and Table 2). Future studies will investigate these candidate compounds to assess their lifespan-extending effects on *C. elegans* in more detail. However, the results of our present study should help elucidate the nutritional mechanism underlying the lifespan-extending effect of BS on *C. elegans*. In conclusion, this study found that the probiotic bacteria CB, EF, and BS extend the lifespan of *C. elegans*. BS has a particularly strong lifespan-extending effect, which suggests that this organism may be useful for new applications in the future. As the present study constituted basic research, the suitability of these bacteria for additional probiotic applications will need to be confirmed in clinical studies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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