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Bacillus Subtilis Delays Neurodegeneration and Behavioral Impairment in the Alzheimer's Disease Model *Caenorhabditis Elegans*

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Abstract. Multiple causes, apart from genetic inheritance, predispose to the production and aggregation of amyloid-β (Aβ) peptide and Alzheimer's disease (AD) development in the older population. There is currently no therapy or medicine to prevent or delay AD progression. One novel strategy against AD might involve the use of psychobiotics, probiotic gut bacteria with specific mental health benefits. Here, we report the neuronal and behavioral protective effects of the probiotic bacterium *Bacillus subtilis* in a *Caenorhabditis elegans* AD model. Aging and neuronal deterioration constitute important risk factors for AD development, and we showed that *B. subtilis* significantly delayed both detrimental processes in the wild-type *C. elegans* strain N2 compared with N2 worms colonized by the non-probiotic *Escherichia coli* OP50 strain. Importantly, *B. subtilis* alleviated the AD-related paralysis phenotype of the transgenic *C. elegans* strains CL2120 and GMC101 that express, in body wall muscle cells, the toxic peptides $A\beta_{3\text{-}42}$ and $A\beta_{1\text{-}42}$, respectively. *B. subtilis*-colonized CL2355 worms were protected from the behavioral deficits (e.g., poor chemotactic response and decreased body bends) produced by pan-neuronal $A\beta_{1\text{-}42}$ expression. Notably, *B. subtilis* restored the lifespan level of *C. elegans* strains that express Aβ to values similar to the life expectancy of the wild-type strain N2 fed on *E. coli* OP50 cells. The *B. subtilis* proficiencies in quorum-sensing peptide (i.e., the Competence Sporulation Factor, CSF) synthesis and gut-associated biofilm formation (related to the anti-aging effect of the probiotic) play a crucial role in the anti-AD effects of *B. subtilis*. These novel results are discussed in the context of how *B. subtilis* might exert its beneficial effects from the gut to the brain of people with or at risk of developing AD.

Keywords: $A\beta_{42}$, Alzheimer's disease, *B. subtilis*, healthy aging, neuroprotection, probiotics, psychobiotics

INTRODUCTION

Alzheimer's disease (AD) is currently the most prevalent neurodegenerative disease worldwide. Every 6 seconds, a new case of AD is diagnosed, and the total number of individuals with AD is expected to increase to 114 million by 2050 [1, 2]. The appearance of the amyloid-β (Aβ) peptide aggregation in the central nervous system (CNS) represents the hallmark of AD, but its etiology is not unique but rather multifactorial and complex [3–6]. There is no current cure or medicine that prevents AD onset or its progression, and currently, only acetylcholinesterase inhibitors, and few other medicines, are being used to alleviate AD symptoms, but not its evolution [2, 7, 8]. There are two important lessons gained

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from the more than 100 failed clinical trials directed against CNS-localized Aβ aggregates. First, an effective AD therapy should include more than one target to decrease the incidence of the multiple risk factors for the onset and progression of the disease. Second, these strategies must be performed at very early stages of the disease, even before neurodegeneration symptoms begin (i.e., as a preventive therapy) [9, 10].

There are two main forms of the disease presentation: genetic (less frequent), in which individuals carry autosomal dominant AD-linked mutations and present clinical symptoms during their sixth or fifth decade of life (or earlier), and sporadic AD, which is not inherited, but multifactorial [3–5, 11] and appears after the seventh decade of age. Sporadic AD represents the most abundant form of the disease (approximately 95% of all cases), and aging constitutes the main risk factor for its onset [12–15]. Earlier, we reported that *Bacillus subtilis*, a human probiotic bacterium forming robust and long-lasting beneficial biofilms [16–18], increases the healthy longevity of the model animal *Caenorhabditis elegans* [19]. This *B. subtilis*-mediated anti-aging effect is mainly funneled through a physiological and reversible downregulation and upregulation of the insulin/insulin growth factor-1 (IGF-1)-like signaling (IILS) and dietary restriction (DR) pathways, respectively [19–21]. Japanese and Jewish centenarians harbor IILS receptor variants (i.e., IGF-1 receptor) with decreased activity, observations that validate the importance of insulin/IGF-1 signaling in lifespan extension and highlight its possible participation in human AD treatment [20, 21].

The existence of a complete neuronal connectivity map and genetic tractability of *C. elegans* make this animal model useful for studying human neurological diseases. Analysis of multiple genetic databases show that a considerable number of human genes associated with AD have a significant homology to *C. elegans* genes, and the genetic tools available for this nematode have allowed the construction of predictive models for studying the molecular mechanism of AD [22, 23]. In this work, we were intrigued to explore the possibility that *B. subtilis* could delay neuronal and behavioral impairments in transgenic *C. elegans* strains used as an AD model [22–27]. The obtained results are discussed through the lens of the possible pathways that *B. subtilis* could use to combat AD onset and progression and the future implementation of this probiotic bacterium in nutraceuticals and functional foods [28–30].

MATERIALS AND METHODS

Strains and growth media

We used the following *C. elegans* strains: wild-type N2 Bristol, the AD model strains CL2006 [dvIs2 (*unc-54*/human Abeta peptide 1-42 minigene) + pRF4], CL2120 [dvIs14 (*unc-54*::beta 1-42 + (pCL26) *mtl-2*::GFP], GMC101 [dvIs100 (*unc-54*p::Abeta-1-42::*unc-54* 3'-UTR + *mtl-2*p::GFP)], and CL2355 [pCL45 (*snb-1*::Abeta 1-42::3' UTR(long) + *mtl-2*::GFP], and the control strain CL2122 [(pPD30.38) *unc-54*(vector) + (pCL26) *mtl-2*::GFP] [24–26]. The used bacterial strains were *E. coli* OP50 and *B. subtilis* NCIB3610 [19]. The AD model nematodes were obtained from the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Nematodes were handled according to standard methods [19, 22]. For all worms, age-synchronized eggs were obtained by incubating embryos from gravid hermaphrodites with bleaching solution (1% NaOCl and 0.25 M NaOH) for 3 min, washing three times, and storing overnight in M9 buffer (22 mM KH_2PO_4, 34 mM K_2HPO_4, 86 mM NaCl, and 1 mM $MgSO_4$) to obtain all animals in stage L1. The L1 population was transferred to Nematode Growth Medium (NGM) agar plates previously seeded with the corresponding bacterial food and incubated until they reached the young adult stage (1-day old L4), approximately 48 h later. Most of the *C. elegans* strains were maintained at 20°C on NGM media seeded with *E. coli* or *B. subtilis* with or without ampicillin (100 μg ml^{-1}) supplementation, respectively [19]. The *C. elegans* CL2355 strain was maintained at 16°C to prevent pan-neuronal Aβ peptide expression [22, 25]. The antifungal amphotericin B (25 μg ml^{-1}; Sigma Co.) was also added to the NGM medium; *E. coli* and *B. subtilis* were grown in Luria-Bertani (LB) broth overnight at 37°C [19].

Analysis of C. elegans aging-related neurodegeneration

Plates were prepared by spreading 50 μl of an overnight culture of *E. coli* OP50 or *B. subtilis* NCIB3610 over the surface of 6-cm diameter plates prepared with NGM agar medium. These plates were incubated overnight at 37°C before seeding with synchronized L1-stage N2 wild-type worms and incubated at 20°C throughout the entire experiment (approximately 30 days). Every 4 days,

the nematodes were labeled with 1,1´-dioctadecyl-3,3,3´,3´,-tetramethylindocarbocyanine perchlorate (DiI, Aldrich), a red fluorescent dye that can fill the worm amphid neurons [31]. The DiI stock solution was 2 mg/ml in dimethyl formamide and was stored at –20°C in a tube wrapped in foil until use. Briefly, OP50- and NCIB3610-fed adult worms of the different ages were spun down, washed, resuspended in 1 ml M9 buffer, and incubated with 5 µl of a 1:200 dilution of DiI stock solution. Incubation was continued on a shaker (75 rpm) for 3 h before spinning, washing, and transferring the labeled worms onto agar pads. To this end, labeled worms were mounted onto a 2% agar pad on a glass slide using 1 M sodium azide (the azide acts as an anesthetic for the worms) and enclosed with a coverslip. Neuron degeneration was examined over time with an Olympus FV1000 laser confocal scanning microscope, and a semi-quantitative analysis was made. The worms were analyzed for the absence of amphid neuron architecture (complete loss), the presence of a complete and intact set of amphid neurons (no loss), or the presence of at least one single structural abnormality, such as wavy, branched, or interrupted dendrites (partial loss) [32, 33]. All experiments were performed at least three times in duplicate.

Culturing bacteria from worms

The N2 *C. elegans* eggs were isolated using a solution of 10% commercial bleach and 1 N NaOH, followed by four washes with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, and 1 M $MgSO_4$). Approximately 500 eggs were transferred to a 60-mm plate with NGM agar and incubated overnight at 20°C with agitation to allow L1 larvae to emerge. Then, approximately 500 L1 larvae per experiment were grown for 48 h on NGM plates (a time that allows worm development to reach the L4 larvae stage) seeded with OP50 *E. coli* cells (1×10^5 cells/plate) or NCIB3610 *B. subtilis* cells (1×10^5 cells/plate). At different incubation times, 50 worms were transferred to Eppendorf tubes containing M9 buffer and 1% Triton X-100. Worms were treated with 25 mM levamisole to induce temporal paralysis, superficially sterilized with 3% commercial bleach for 15 min and washed three times with M9 buffer. After the worms were surface-sterilized, worms devoid of outside bacteria were disrupted using a pellet pestle (Sigma Co.), centrifuged, and resuspended in 500 µl M9 buffer. Finally, 50 µl of each cell suspension were used to prepare serial dilutions of the bacteria before counting. To this end, 100 µl of the appropriate serial dilutions was spread with a Drigalski scraper on LB Petri dishes. The number of colony-forming units (CFUs) was determined after 24 h of incubation at 37°C.

Octanol and diacetyl (DA) time response assays

For the behavioral experiments, *C. elegans* N2 worms were fed on OP50 or NCIB3610 bacterial cells from the L1-larval stage to adulthood at 20°C. Repulsion and attraction behavioral assays using octanol (1-octanol, Sigma-Aldrich) or DA (butane-2,3-dione, Merck) as repellent or attractant agents, respectively, were performed as previously described [25, 26]. Briefly, OP50- or NCIB3610-fed adult worms of different ages were washed three times with M9 buffer to remove any residual bacteria and placed in NGM plates without food. One hour after food starvation, for the repellent assay, a paintbrush hair previously dipped in 100% undiluted octanol was placed in front of a moving animal (care was taken to not touch the nematode). The octanol response time was scored as the time (s) from presentation to the initiation of a backward or escape movement. Sterile water was used instead of octanol as a control, and assays were halted at 20 s to account for spontaneous reversals (data not shown). For the attractant assay, 1 h after food starvation, a 1-µL drop of 0.5% DA in ethanol was placed 1.5 cm in front of a moving animal (without touching it). The DA response time was scored as the time (s) from presentation to the initiation of a forward movement in the direction to DA. Ethanol was used instead of DA as a control (data not shown). All experiments were performed in triplicate.

Chemotaxis index (CI) assays

The OP50- or NCIB3610-fed N2 worms were collected, washed three times with M9 buffer, and seeded in NGM 10-cm plates without food for 1 h. Then, approximately 75 worms were placed in the center of 6-cm plates prepared with 2% agar, 1 mM $CaCl_2$, 1 mM MgSO4, and 25 mM phosphate buffer (pH 6.0). After all animals were transferred to the center of the assay plates, 2 µl of attractant were seeded 2 cm from the center of the plate, and 2 µl of solvent (control) in which the attractant was diluted were seeded equidistantly. Both the attractant and the control were added with a 1-µl drop of 1 M azide. The plates were

incubated for 1 h at 20 or 23°C (as indicated in the legend figures). Then, worms found at each end of the plates were counted, and the CI was calculated. The attractant compounds used for the assays were 0.5% DA diluted in ethanol and 0.1% isoamyl alcohol (IAA; Sigma-Aldrich) diluted in water and NaCl 150 mM. The CI is defined as the number of worms at the attractant or repellent location – number of worms at the control location divided by the total number of worms on the plate [24].

Paralysis assay

The CL2122, CL2120, and GMC101 L1 larvae were cultured at 20°C on OP50 or NCIB3610 bacterial lawns until adulthood (L4 stage). Then, 1-day old L4 adults were transferred to NGM 6-cm plates without bacteria. After 1-h food starvation, the 6-cm plates seeded with worms were shifted to 25°C, and paralysis was scored each day until the last worm became paralyzed. Nematodes were considered paralyzed if they failed to complete a full body movement or only moved their head when gently touched with a platinum wire [26].

Behavioral assays of C. elegans AD models

The CL2122 and CL2355 L1 larvae were fed on OP50 or NCIB3610 bacterial cells at 16°C until they reached the L3-larval stage (approximately 36 h). The L3 larvae were shifted to 23°C (to express the Aβ peptide) and incubated for another 36 h until adulthood (L4 stage). For the chemotaxis assays, approximately 250 L4 larvae were collected, washed three times with M9 buffer, and seeded on NGM 10-cm plates without food for 1 h. Then, approximately 100 worms were placed on the center of 6-cm plates prepared with 2% agar, 1 mM $CaCl_2$, 1 mM MgSO4, and 25 mM phosphate buffer (pH 6.0). After all animals were transferred to the center of the assay plates, 2 µl chemoattractant (0.5% DA in 95% ethanol), along with 1 µl 1 M sodium azide, were added to the original spot. On the opposite side of the attractant, 1 µl sodium azide and 2 µl ethanol (control) were added. Assay plates were incubated at 23°C for 1 h, and the CI was calculated as indicated. For the body bend assay, OP50- and NCIB3610-fed L4 worms were collected, washed three times with M9 buffer, and seeded on NGM 10-cm plates without food for 1 h. Each worm was then transferred to a single well of a 24-well plate with 1.5 ml M9 buffer. After allowing adaptation for 20 s, worms were scored for the number of body bends generated in 30 s. A body bend was defined as a change in the direction of propagation along the y-axis, assuming that worms were travelling along the x-axis [25]. Twenty worms of each group were evaluated, and all experiments were performed three times in duplicate.

Lifespan assays

Lifespans for *C. elegans* N2 and AD strains were monitored at 20 or 23°C as described previously [19]. Briefly, embryos were isolated by exposing hermaphrodite adult worms to alkaline hypochlorite treatment for 3 min, processed as indicated above, and synchronized eggs were allowed to develop. In all cases, L4/young adult worms ($n = 100$) were used at time zero for lifespan analysis; they were transferred to fresh plates previously seeded with OP50 or NCIB3610 bacterial cells each day until the assay was completed. Worms were considered dead when they ceased pharyngeal pumping and did not respond to prodding with a platinum wire. Worms with internal hatching were removed from the plates and excluded from lifespan calculations. All experiments were repeated at least three times in duplicate.

Statistical analysis

All assays were performed at least three times in duplicate. Mean survival days, standard error of the mean (S.E.M.), intervals of mean survival days with 95% confidence, and equality p values to compare averages were calculated by log-rank and Kaplan-Meier tests using the OASIS program. The S.E.M. values are used in the figures; $p < 0.5$ was considered statistically significant.

RESULTS

Bacillus subtilis delayed age-related neurodegeneration and cognitive damage in C. elegans

We fed young adult N2 wild-type *C. elegans* (1-day-old adult worms) with the regular bacterial food, the OP50 *E. coli* strain, or the probiotic *B. subtilis* strain NCIB3610, and investigated the *in vivo* effect of *B. subtilis* on neural deterioration retardation throughout the life time (lifespan expectancy) of both worm populations (Fig. 1A). Bacteria that are

disrupted by the worm grinder and those that survive and reach the intestine constitute the worm food and the worm gut flora, respectively (Fig. 1B) [19]. Age-related neurodegeneration [32, 33] was verified by the formation of neuronal defects such as beaded, wavy, branched, and/or interrupted dendrites or soma branching in OP50- or NCIB3610-colonized worms. To this end, OP50- and NCIB3610-fed worms of different ages (Fig. 1A) were stained with the fluorescent dye DiI, which specifically labels amphid (and phasmid; not shown) neurons to highlight the chemosensory structures of live nematodes [31, 34]. The staining of OP50-colonized young adult worms (4 days old) with Dil revealed the complete integrity of the neuronal network (i.e., normal amphid channels and nerve ring structures, dendrites and sensory neurons, respectively) (Figs. 1C and 2A, 4 days old, column and left panel, respectively). As the OP50-colonized worm population aged (up to 12 days of cultivation), we observed different defects (partial neural loss) in the amphid chemosensory structures in approximately 40 and 60% of the worm population (Figs. 1C and 2B, 8 and 12 days old, columns and upper panel, respectively). From that age on (12 days onwards), we started to observe signs of total neural deterioration (i.e., neuronal death) in the OP50-colonized worm population. The corresponding percentages of partial/total neural deterioration, at the ages of 16 and 20 days, were 60%/10% and 30%/70%, respectively (Figs. 1C and 2C, 16 and 20 days old, columns and left panels, respectively). After 24 days of cultivation, the OP50-colonized worms showed a complete loss of neuronal architecture, Fig. 1C (24 days old column). Interestingly, for worms fed on the probiotic strain NCBI3610, the 37% gained in lifespan extension when compared with the lifespan of worms colonized by OP50 cells ($p < 0.001$, Fig. 1A) correlated with a notable delay in neuronal deterioration. In contrast to the 40% of the 8-day-old OP50-colonized worms, the 100% of NCIB3610-colonized worms of the same chronological age retained a completely normal neuronal architecture (Figs. 1D and 2A, 8 days old, column and right panel, respectively). At longer incubation times (12 days old and beyond), the differences in neuronal architecture preservation between OP50- and NCIB3610-colonized worms of the same chronological age became more notorious. While 20-day-old OP50-colonized worms showed percentages of normal, partial, and total neuronal deterioration of 0, 30, and 70%, respectively (Fig. 1C, 20 days old column), NCIB3610-colonized worms of the same chronological age showed percentages of 40, 50, and 10%, respectively (Figs. 1D and 2A, 20 days old, column and bottom panel, respectively). At advanced chronological ages (i.e., 24 and 28 days), when the neuronal architecture of OP50-colonized worms was completely damaged, the NCIB3610-colonized population showed a proportion of worms with complete neuronal loss (Fig. 2C, right panel), but still contained a significant proportion of worms with normal or partial neuronal architecture, Fig. 1D (24 and 28 days, columns). The presented results (Figs. 1 and 2) demonstrate that the neuronal architectural decay of worms of the same chronological age can markedly differ in function of the type of bacterium (i.e., probiotic or non-probiotic) that colonized their guts, strongly suggesting that behavioral responses are also affected differently.

To correlate the morphological neuroprotective effect of *B. subtilis* on the functionality of the sensory apparatus of *C. elegans* throughout adult life (Fig. 3A), we performed behavioral chemotaxis tests in similarly aged worms colonized by NCIB3610 or OP50 bacteria. The chemotaxis response in *C. elegans* is mediated by the interplay of several sensory neurons and interneurons to stimulate the motor neurons so that the individual approximates or avoids a certain chemical signal (an attractant or a repellent, respectively) [19, 34]. As compared with OP50-colonized worms, *B. subtilis*-colonized worms displayed an enhanced behavioral response (improved response times) when confronted with negative and positive environmental inputs (avoidance or attraction to harmful or attractant signals; Fig. 3B and 3C, respectively). Overall, the lower (more rapid) response times of *B. subtilis*-colonized worms, compared with OP50-colonized worms, to different external stimuli (Fig. 3B, C) correlated well with the CIs measured at different chronological ages (16, 20, 24, and 28 days old; Figs. 3D–F). For instance, the CIs of 16-day-old elderly OP50- or NCIB3610-colonized worms to DA, IAA, and NaCl were 0.18 ± 0.02 and 0.39 ± 0.04; 0.16 ± 0.02 and 0.33 ± 0.03; 0.20 ± 0.02 and 0.40 ± 0.04, respectively ($n = 75$, $p < 0.001$; Fig. 3D–F), The improved behavioral performance (i.e., higher CIs) of NCIB3610-colonized worms, compared with OP50-colonized worms, remained during the complete adult life of both compared worm populations. Even at a very old age (i.e., 28 days old), when all OP50-colonized worms were dead, the NCIB3610-colonized worms showed a behavioral response significantly better (CIs of 0.14 ± 0.04, 0.12 ± 0.02,

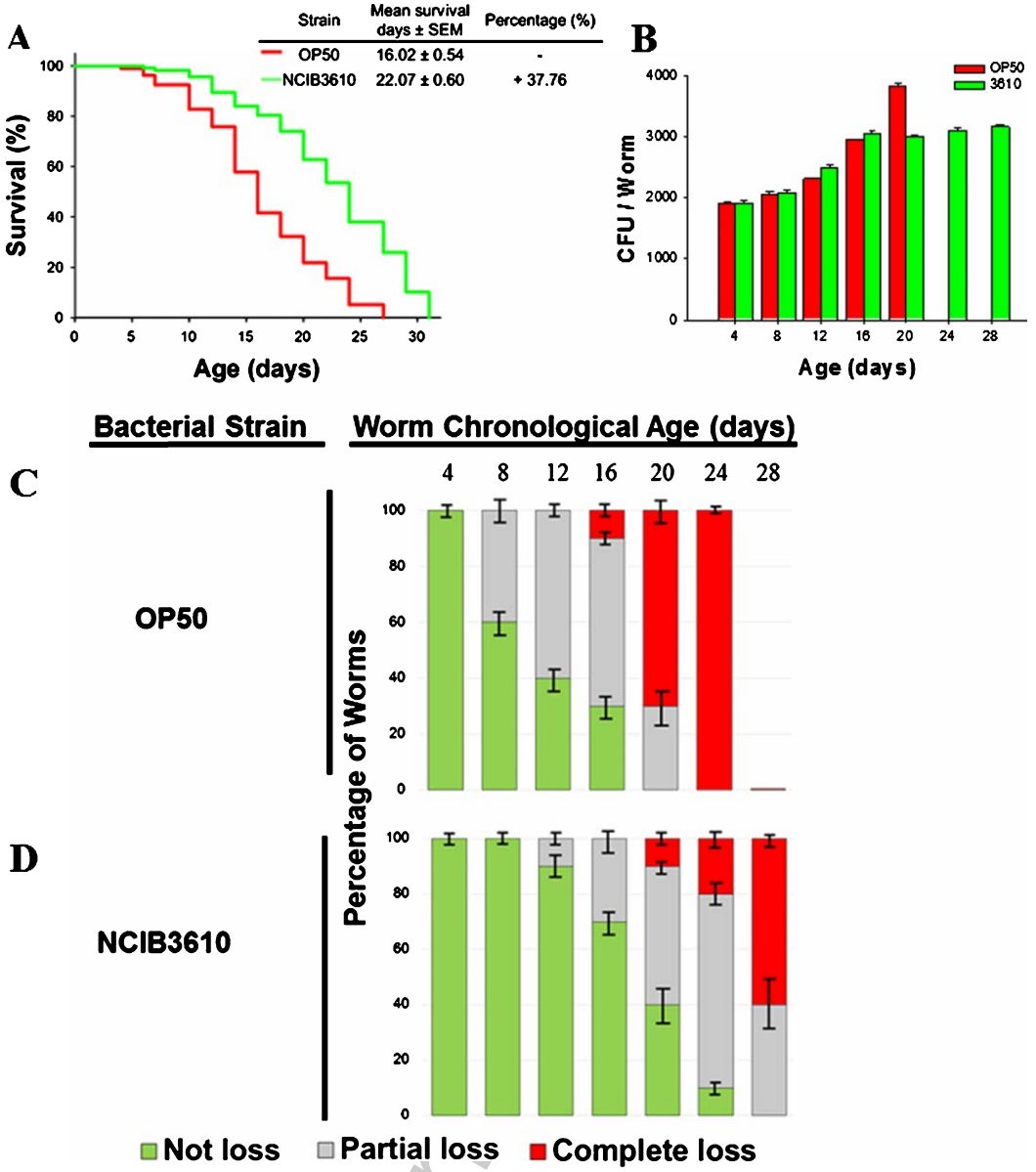


Fig. 1. Age-related neuroprotection by *B. subtilis*. A) Life expectancy of *C. elegans* that harbored probiotic or non-probiotic bacteria in the intestine. One hundred young-adult (1-day old) wild-type Bristol strain N2 worms were fed on *E. coli* OP50 or probiotic *B. subtilis* NCIB3610 bacteria (red and green, respectively). Worms were grown on bacteria-seeded 10-cm NGM agar plates at 20°C, and survival was monitored as indicated until the last worm died (see Materials and Methods for details). The life expectancy of NCIB3610-colonized worms was 37% longer than the lifespan of OP50-colonized worms ($p < 0.001$). A typical output of three independent experiments performed in duplicate is presented. B) Worm intestine colonization by OP50 or NCIB3610 bacteria. L4 worms were allowed to develop at 20°C on NGM agar plates seeded with OP50 *E. coli* or NCIB3610 *B. subtilis* cells (red and green bars, respectively), as indicated in Material and Methods. At each of the indicated ages, 50 worms were transferred to Eppendorf tubes, superficially sterilized, and disrupted before counting the number of *E. coli* or *B. subtilis* cells in the worm gut. The data are representative of at least three independent experiments. Error bars show the mean ± SEM from at least three independent experiments. See Material and Methods for details. C, D) Semi-quantification of age-related neurodegeneration. Ten OP50- or NCIB3610-colonized N2 worms (C and D, respectively), grown on NGM plates at 20°C, were taken at the indicated times, processed, and labeled with DiI, as indicated in Materials and Methods, to determine the grade of age-related neuronal deterioration (no loss, partial loss, or total loss). See Materials and Methods for details. Results are expressed as a percentage of initial worm population ($n = 100$) ± S.E.M.

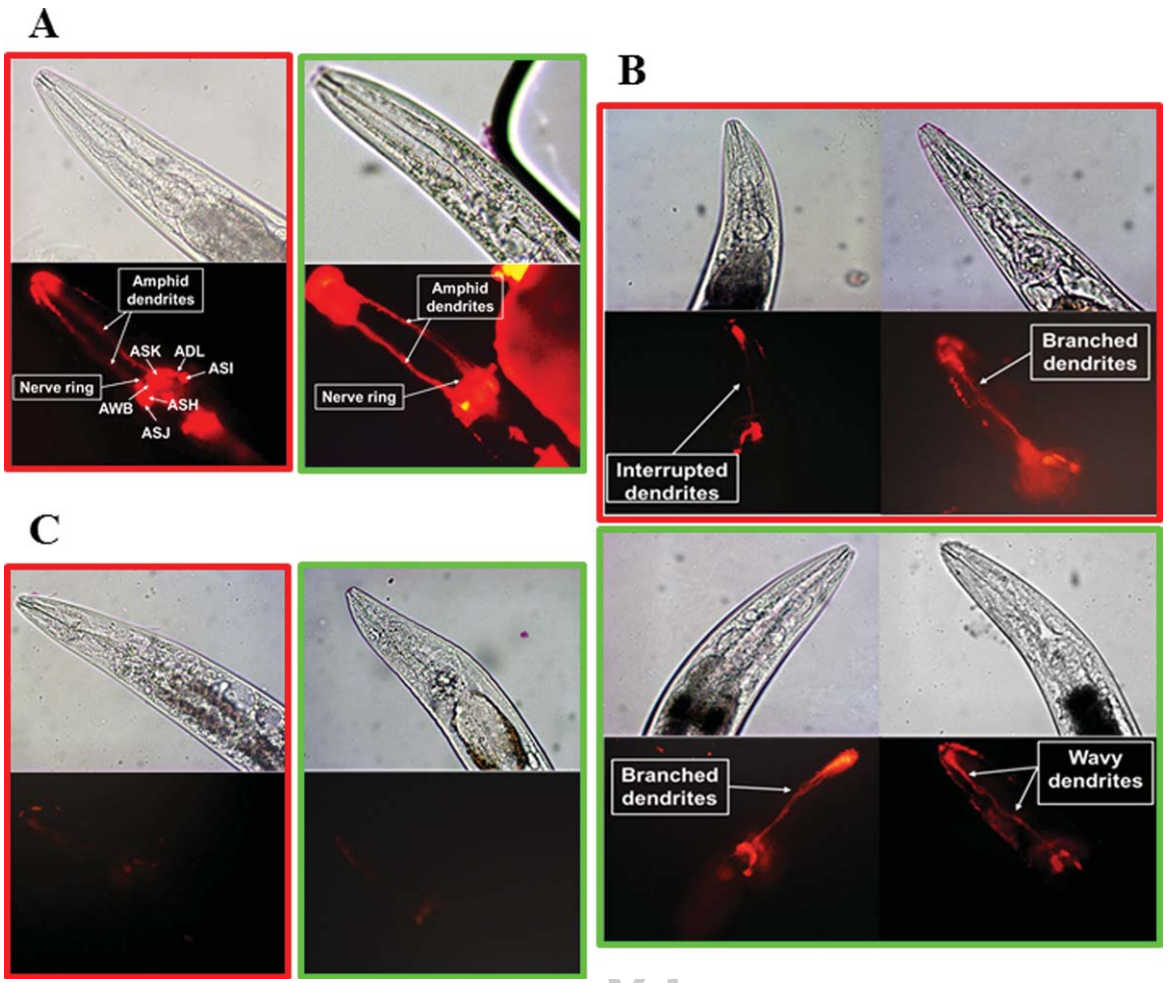


Fig. 2. Neuronal morphological changes of aging worms colonized by OP50 or NCIB3610 bacteria. Aging N2 worms, colonized by OP50 or NCIB3610 bacterial cells (red and green rectangles in the figure, respectively) at different ages, were labeled with fluorescent DiI to highlight amphid neuron morphology: normal morphology or no neuronal loss, A; partial neuronal alterations or partial neuronal loss, B; and total neuronal deterioration or total neuronal loss, C. See Materials and Methods for details. Worm ages are as follow: 4 days old and 8 days old, A; 8 days old and 20 days old, B; and 20 days old and 28 days old, C; for OP50-colonized or NCIB3610-colonized worms, respectively. The top and bottom micrographs (phase contrast and fluorescence microscopy, respectively) in A to C are representative of 10 independent worm images analyzed for each age. Arrows in A indicate the location of the chemosensory worm neurons (i.e., ASK, ADL, ASI, ASH, ASJ, AWB), and arrows in B indicate some of the age-associated neuronal alterations.

and 0.20 ± 0.02; for DA, IAA or NaCl, respectively; $n = 75$, $p < 0.1$) than that of the 20-day-old OP50-colonized worms (Fig. 3D–F).

The overall results (i.e., delayed aging, neuroprotection, improved behavioral responses, Figs. 1–3), and the knowledge that aging and neurodegeneration are important risk factors for AD development [13, 14, 35, 36], prompted us to use several transgenic *C. elegans* strains that express the human Aβ peptide to investigate whether *B. subtilis* might represent a new alternative against the disease.

Bacillus subtilis alleviated the paralysis phenotype of transgenic C. elegans expressing the human Aβ peptide in muscle

Caenorhabditis elegans offers a valuable platform for investigating the cellular and molecular mechanisms of AD [22, 23]. The Aβ is believed to be the major cause of AD pathogenesis, and its expression in transgenic *C. elegans* strains produces several pathological features important to better understand AD pathology [24, 26, 37, 38]. Two of the transgenic AD

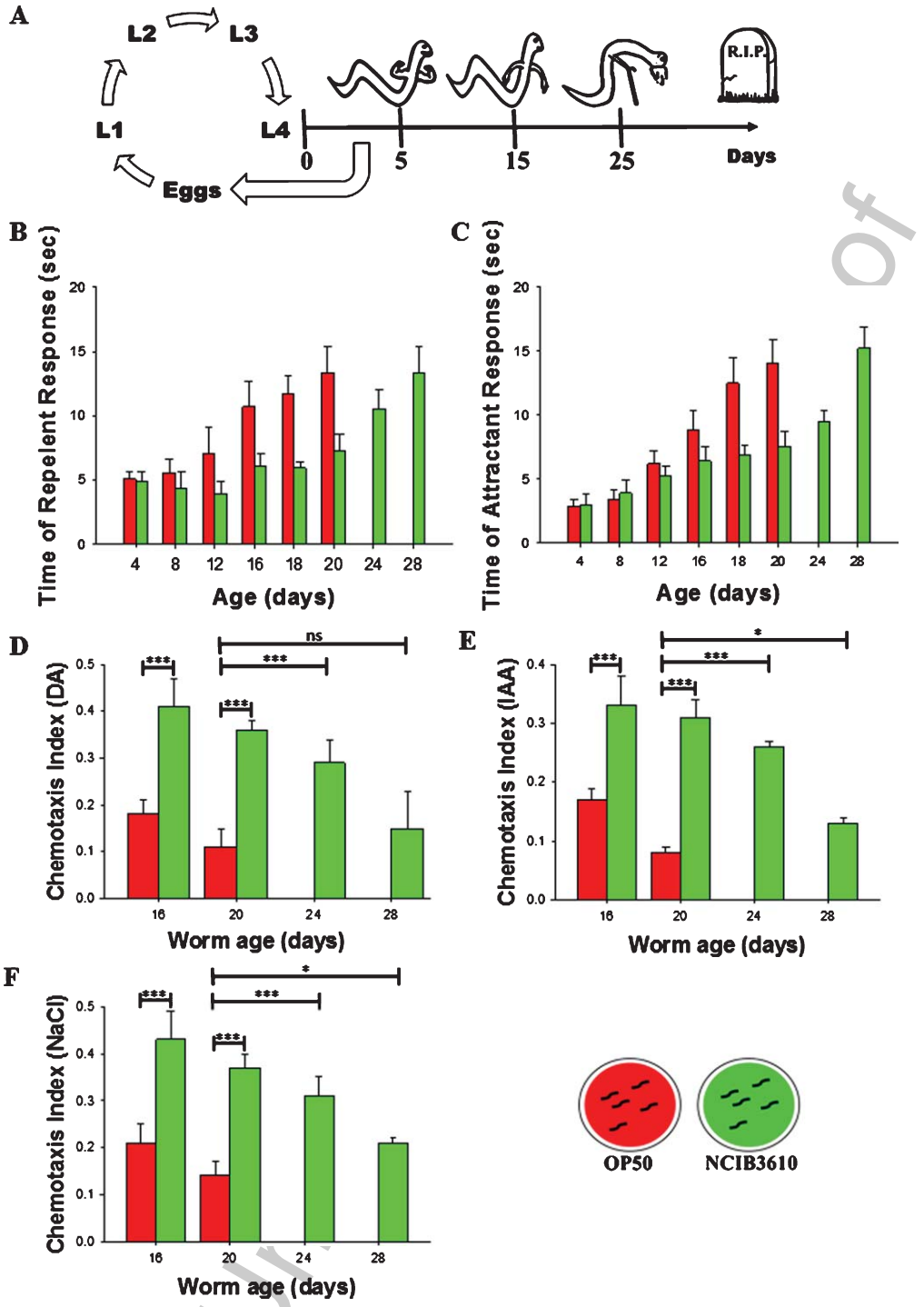


Fig. 3. *B. subtilis*-mediated cognitive improvement during *C. elegans* aging. A) Schematic representation of C. elegans life cycle from egg-laying to adult worm death. B, C) Average response times (in seconds, sec, y-axis) of OP50- and NCIB3610-colonized N2 worms (red and green colors, respectively) of different ages (in days, x-axis) to repellent (octanol, B) and attractant (diacetyl [DA], C) exposition (see Materials and Methods for details). Results represent the mean ± S.E.M of three independent experiments performed in duplicate. D-F) Chemotaxis index of N2 worms of different ages exposed to different attractants: 0.5% DA (D), 0.1% isoamyl alcohol (IAA, E), and 150 mM NaCl (F). A typical result from one of the three independent experiments performed in duplicate is presented (mean ± S.E.M). Asterisks indicate statistical significance (***$p < 0.001$; **$p < 0.01$; and *$p < 0.1$; ns, no significant difference, $p > 0.5$).

C. elegans strains are CL2120 and GMC101, which express human Aβ peptides of different sizes and toxicities, namely $A\beta_{3\text{-}42}$ and $A\beta_{1\text{-}42}$, respectively [24, 26]. In the *C. elegans* strain CL2120, the $A\beta_{3\text{-}42}$ peptide is constitutively expressed under the control of the *unc-54* promoter in body wall muscle cells and produces a chronic and progressive paralysis phenotype [24]. In the first set of the performed paralysis experiments, young larvae (L1) of the CL2120 strain and its wild-type control CL2122 strain were fed on *E. coli* OP50 or *B. subtilis* NCIB3610 cells for 48 h at 20°C until they reached adulthood (L4 stage). They were then washed several times and starved from bacterial food for 1 h (Fig. 3A). The starved young adult worms, which contained OP50 or NCIB3610 bacteria colonizing their guts, were shifted to 25°C and paralysis was recorded. Worms that did not move or only moved the head (under a gentle touch with a platinum loop) were scored as paralyzed (see Materials and Methods for details). The control CL2122 worms, maintained at 25°C, displayed a motile (no paralysis) phenotype for the duration of the experiment (over 1 week after adulthood), regardless of the gut bacteria (OP50 or NCIB3610) they harbored (Fig. 3B). However, the human Aβ-peptide-expressing strain CL2120, colonized by the OP50 *E. coli* strain, displayed an age-dependent paralysis phenotype that started 2 days after the temperature increase from 20 to 25°C, and 4 days after the temperature upshift, the entire OP50-colonized CL2120 worm population was paralyzed (Fig. 3C). Comparatively, when the CL2120 worms were colonized by *B. subtilis* NCIB3610, 100% of the worm population were protected from paralysis and remained fully motile during the experiment (over 1 week after adulthood; Fig. 3C). The CL2120 strain expresses a less-toxic form of the human Aβ peptide (i.e., $A\beta_{3\text{-}42}$), and therefore, the paralysis phenotype observed in this transgenic strain is ameliorated [24]. By contrast, the GMC101 strain expresses the full-length human Aβ peptide ($A\beta_{1\text{-}42}$), and thus, the paralysis phenotype displayed in this transgenic worm is more severe [26]. In order to confirm the CL2020 strain results, we performed a second set of paralysis experiments in the GMC101 strain colonized by OP50 or NC1B3610 bacteria. As shown in Fig. 3D, the paralysis phenotype in the OP50-colonized GMC101 strain was detected more rapidly and was more severe than the observed paralysis displayed by the OP50-colonized CL2120 strain (Fig. 3C). Indeed, almost 90% of the OP50-colonized GMC101 worms were completely immotile (paralyzed) 2 days after the temperature increase (Fig. 3D). Intriguingly, *B. subtilis* NCIB3610 significantly delayed the start and severity of paralysis in GMC101 worms (Fig. 3D). While the paralysis of the OP50-colonized GMC101 worm population was almost total (100%) 2 days after the temperature increase from 20 to 25°C, almost 97% of the NCIB3610-colnized GMC101 worm population were not paralyzed. Furthermore, only 15% of NCIB3610-colonized GMC101 worms were immotile (paralyzed) after 3 days of the temperature upshift, compared with the 100% of OP50-colonized worms that were immotile at that time (Fig. 3D). The PT_{50}, the time interval from the onset of paralysis at which 50% of the worms were paralyzed, in GMC101 worm populations was 1.7 ± 0.3 days ($n = 75$) and 4.6 ± 0.5 days ($n = 75$; $p < 0.001$) for OP50- and NCIB3610-colonized worms, respectively. Thus, at the assayed times, there was complete paralysis prevention or significant amelioration in transgenic worms that express the less severe and the more toxic forms of the human Aβ peptide, $A\beta_{3\text{-}42}$ or $A\beta_{1\text{-}42}$, respectively, when *B. subtilis* colonized the worm intestine (Fig. 3C, D).

Bacillus subtilis alleviated behavioral deficits of transgenic C. elegans expressing pan-neuronal Aβ peptide

Transgenic *C. elegans* individuals with neuronal human Aβ peptide expression show learning-deficit behavioral phenotypes [25]. The *C. elegans* strain CL2355 employs the synaptobrevin promoter (*snb-1*) to drive pan-neuronal human Aβ peptide expression (*snb-1*::$A\beta_{1\text{-}42}$) after a temperature increase to 23°C [25]. We consider this transgenic AD strain to be a useful tool to evaluate the protective effect of *B subtilis* on the deteriorated behavioral performance of transgenic worms with neuronal Aβ expression. One sensory behavior we examined in this strain was chemotaxis. The age-synchronized wild-type control strain (CL2122) and transgenic CL2355 *C. elegans* were cultured at 16°C from egg hatching, using *E. coli* OP50 or *B. subtilis* NCIB3610 as a food source up to reaching the L3 larval stage, and then shifted to 23°C for 36 h to induce the production of pan-neuronal Aβ peptide while the final larval stage (L4) was reached (Fig. 4A). These young adult L4 CL2355 and CL2122 worms were starved from food (OP50 or NCIB3610) for 1 h before to compare their chemotactic response toward the attractant DA (Fig. 4A). As shown in Fig. 4B, OP50-colonized CL2355 worms exhibited a poor

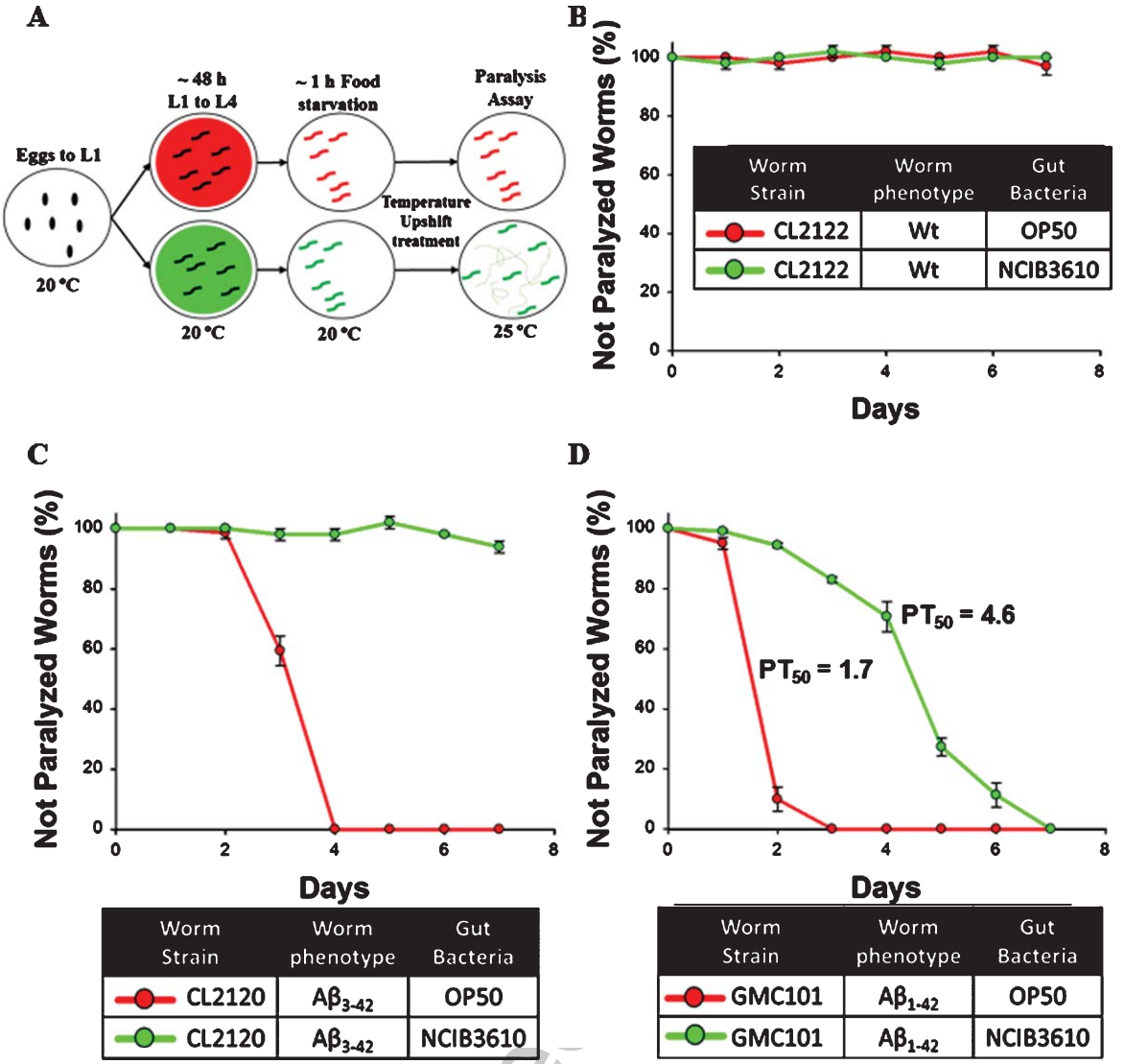


Fig. 4. *B. subtilis* protected against Aβ-induced progressive paralysis in *C. elegans* AD model strains. A) A cartoon that summarizes the paralysis assay performed on OP50- and NCIB3610-colonized AD model worms (red and green colors, respectively; see Materials and Methods for details). B-D) Percentages of paralyzed CL2122 wild-type (wt) worms (control, B), and AD model CL2120 and GMC101worms (C and D, respectively) fed on OP50 or NCIB3610 bacteria as indicated. PT_{50}, in panel D, indicates the time in which 50% of the worm population were paralyzed. Nematodes were considered paralyzed if they failed to complete a full body movement or only moved their head when gently touched with a platinum wire. Paralysis was scored every day until the last worm became paralyzed. Panels B-D show a representative result from three independent experiments performed in duplicate (mean ± S.E.M).

chemotactic response toward DA (CI = 0.19 ± 0.01; $n = 100$) compared with the OP50-colonized CL2122 control strain (CI = 0.55 ± 0.04; $n = 100$, $p < 0.001$). Importantly, NCIB3610-colonized CL2355 worms displayed a chemotactic response toward the attractant (CI = 0.62 ± 0.04; $n = 100$) that was indistinguishable from the chemotactic response of the control wild-type CL2122 strain colonized by OP50 bacteria (CI = 0.65 ± 0.04, $n = 100$; Fig. 4B).

We also measured whether *B. subtilis* improved the slowed locomotion response (body bends) that occurs in worms that express pan-neuronal Aβ peptide (Fig. 4C) [22, 25]. The OP50-colonized CL2355 worms exhibited a low number of body bends (50 ± 4; $n = 100$) compared with the body bend number from the OP50-colonized control strain CL2122 (85 ± 6; $n = 100$, $p < 0.001$). Importantly, NCIB3610-colonized CL2355 worms displayed a

body bend response (98 ± 6 body bends; $n = 100$) also indistinguishable from the motility response of the control wild-type CL2122 strain colonized by OP50 cells (100 ± 6 body bends; $n = 100$; Fig. 4D). These results show that the cognitive impairments (deleterious behavioral responses) for food detection (Fig. 4B) and locomotive activity (Fig. 4D) produced by pan-neuronal human Aβ peptide expression are eliminated by *B. subtilis*.

Bacillus subtilis restored the healthy lifespan of Aβ peptide-expressing C. elegans strains

The life expectancy of transgenic *C. elegans* expressing human Aβ peptide is shortened [25, 38]. To evaluate whether the protective effect of *B. subtilis* on neurodeterioration and behavioral impairment of transgenic AD *C. elegans* was also translated to life expectancy, we performed lifespan assays in the AD strains CL2006, CL2120, and CL2355 colonized by OP50 or NCIB3610 bacterial cells [19]. First, we compared the lifespan of the transgenic CL2006 and CL2120 strains that constitutively express human Aβ when colonized by *B. subtilis* NCIB3610 or *E. coli* OP50 (see Material and Methods for details) [22]. In parallel, we compared the lifespan values of the AD strains CL2006 and CL2120, colonized by OP50 or NCIB3610 cells, with the lifespans of the corresponding control strains wild-type N2 and CL2122, respectively. The lifespan expectancy of the OP50-colonized CL2006 and CL2120 AD worms decreased by 26 and 29%, respectively, compared with the lifespan of OP50-colonized wild-type worms (Fig. 6A, B, $n = 100$, $p < 0.001$). Interestingly, *B. subtilis* NCIB3610 robustly extended the lifespan of both AD strains, CL2006 and CL2120, to a level indistinguishable of the life expectancy (mean lifespan value of ∼16 days) of the corresponding OP50-colonized N2 and CL2122 wild-type worms, respectively (Fig. 6A, B, $n = 100$, $p < 0.001$). In the case of the AD model strain CL2355, which produces a pan-neuronal expression of the human Aβ peptide, its life expectancy when colonized by OP50 cells was severely reduced (∼40% decrease) compared with the lifespan level of the control wild-type strain CL2122 (Fig. 6C, $n = 100$, $p < 0.001$). Attractively, the probiotic bacterium significantly increased the lifespan of CL2355 worms (from ∼9 days to ∼12 days), although not exactly to the same level (∼15 days) of the corresponding OP50-colonized wild type CL2122 strain (Fig. 6C, $n = 100$, $p < 0.001$).

DISCUSSION

Caenorhabditis elegans and mammalian neurons are remarkably similar in terms of functionality and connectivity, and *C. elegans* offers a valuable and simple tool to unravel what might be happening in the aging mammalian brain under normal and pathological conditions [20, 23, 27, 34]. The results presented in this work show that *B. subtilis* can delay neuronal aging (Figs. 1–2) and improve behavioral responses in elderly wild-type worms (Fig. 3). Since aging is the main risk factor for AD development [2, 13, 15, 36], we investigated whether the anti-aging effect of this bacterium [19, 39] would also protect against AD. In particular, we measured the deleterious effects of Aβ peptide expression in transgenic worms that harbor the probiotic bacterium in their guts. *Bacillus subtilis* stopped or delayed paralysis in the AD transgenic strains CL2120 and GMC101, respectively (Fig. 4). The *C. elegans* CL2120 strain, which constitutively expresses a less-toxic version of the Aβ peptide ($A\beta_{3\text{-}42}$) in wall muscle cells, exhibited a chronic, albeit smoother paralysis progression (Fig. 4C) than the more toxic version of the Aβ peptide ($A\beta_{1\text{-}42}$) expressed by the GMC101 strain (Fig. 4D). Accordingly, *B. subtilis* significantly improved the behavioral responses of transgenic CL2355 *C. elegans* with pan-neuronal Aβ peptide distribution (Fig. 5) and extended the lifespan in AD model worms to levels similar to those observed in wild-type animals (Fig. 6).

Bacillus subtilis is a probiotic member of the human gut microbiota [40–45]. Probiotics are live microorganisms (principally bacteria) which, when consumed in adequate quantities, have beneficial health effects on consumers [46, 47]. Recently, a new probiotic category was proposed: psychobiotics (i.e., probiotics that benefit behavior and combat neuronal disorders) [48]. Psychobiotics modulate brain functions through the gut-brain-axis [49, 50]; they can alter the gut microbiota composition [51], influence immune-neuron system communication, modify host-produced neurotransmitters, and/or synthesize neurotransmitters *de novo* [52–54]. The failure of the more than 100 AD clinical trials with drugs that target CNS Aβ aggregates leads researchers and clinicians to consider other hypotheses and therapies [4–6, 55–57]. Could pro(psycho)biotics be used in AD patients? In a recent report, probiotic lactic bacteria (LBA) taken daily over a short time (12 weeks) produced a moderate, but significant improvement in some metabolic statuses and the Mini-Mental

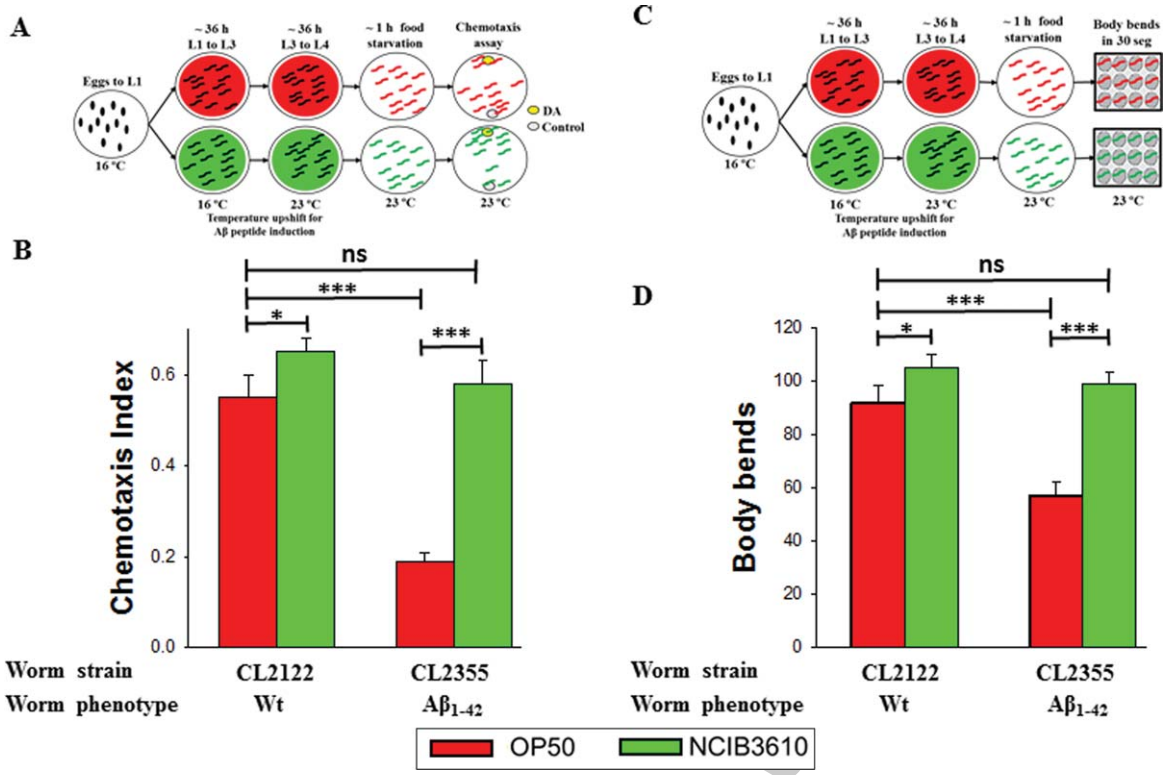


Fig. 5. *B. subtilis* improved the behavioral response of *C. elegans* expressing pan-neuronal Aβ aggregates. A) and C) Cartoons that summarize the chemotactic (B) and basal slowing movement (C) assays of OP50- and NCIB3610-colonized AD CL2355 worms (red and green colors, respectively; see Materials and Methods for details). B and D) Chemotaxis indices for CL2122 (control or wild-type, wt) and CL2355 (AD model) worms to 0.5% diacetyl (DA, attractant, B), and body bends of CL2122 (control) and CL2355 (AD model) worms scored for 30 s, as shown in panels A and C, respectively. A typical result out of three independent experiments (performed in duplicate) is presented (mean ± S.E.M). Asterisks indicate statistical significance (*$p < 0.1$; ***$p < 0.001$; ns, no significant difference, $p > 0.5$).

State Examination scores of elderly 60-to-95-year-old male and female AD patients [58]. This study showed that the gut microbiome, inhabited by trillions of microorganisms, can be modulated by dietary interventions with probiotic (psychobiotic) bacteria to combat AD [59, 60].

Could *B. subtilis* be used in the future to delay or treat human AD? If so, how might anti-AD *B. subtilis* work? Unfortunately, our understanding of AD is incomplete, most likely because most of the information about the disease etiology comes from familial (genetic-mutation-related) AD, which represents a minor proportion of AD cases [1, 13]. However, AD etiology is multifactorial and complex; it involves multiple distinct and overlapping redundant pathways of neuronal damage [4, 8, 12, 14, 15, 61]. In this sense, one common feature of the failed clinical trials is that, regardless of their individual targets, all of them were based on the belief that AD pathology emanates from a single protein: the Aβ peptide (i.e., the amyloid cascade hypothesis) [3, 55]. Therefore, if *B. subtilis* only targets Aβ, it would not likely constitute a valuable therapeutic tool for AD. However, we envision at least three different (but overlapping and simultaneous) possible scenarios of how *B. subtilis* might be employed as a gut-member to delay or treat AD in the future [5, 23, 29, 48, 49, 51, 62] (Fig. 7).

Evidently, one weapon that *B. subtilis* would use to fight AD is the anti-aging effect of the bacterium [19, 39, 63]. There are two main genetic pathways, evolutionary conserved from nematodes and flies to human beings, that control the aging process in living organisms: dietary restriction (DR) and the insulin/insulin growth factor-1 (IGF-1)-like signaling (IILS) system [19–21, 39]. In *C. elegans*, the IILS pathway is under the control of the nutrient-related signal receptor DAF-2, that is the homologue of the human insulin-like receptor IGFR that negatively regulates DAF-16 and HSF-1 [19–21, 39]. Here, DAF-16 (homologue to the FOXO human transcription factor) and HSF-1 (heat shock factor) play a crucial role in the expression of numerous genes involved in lifespan extension

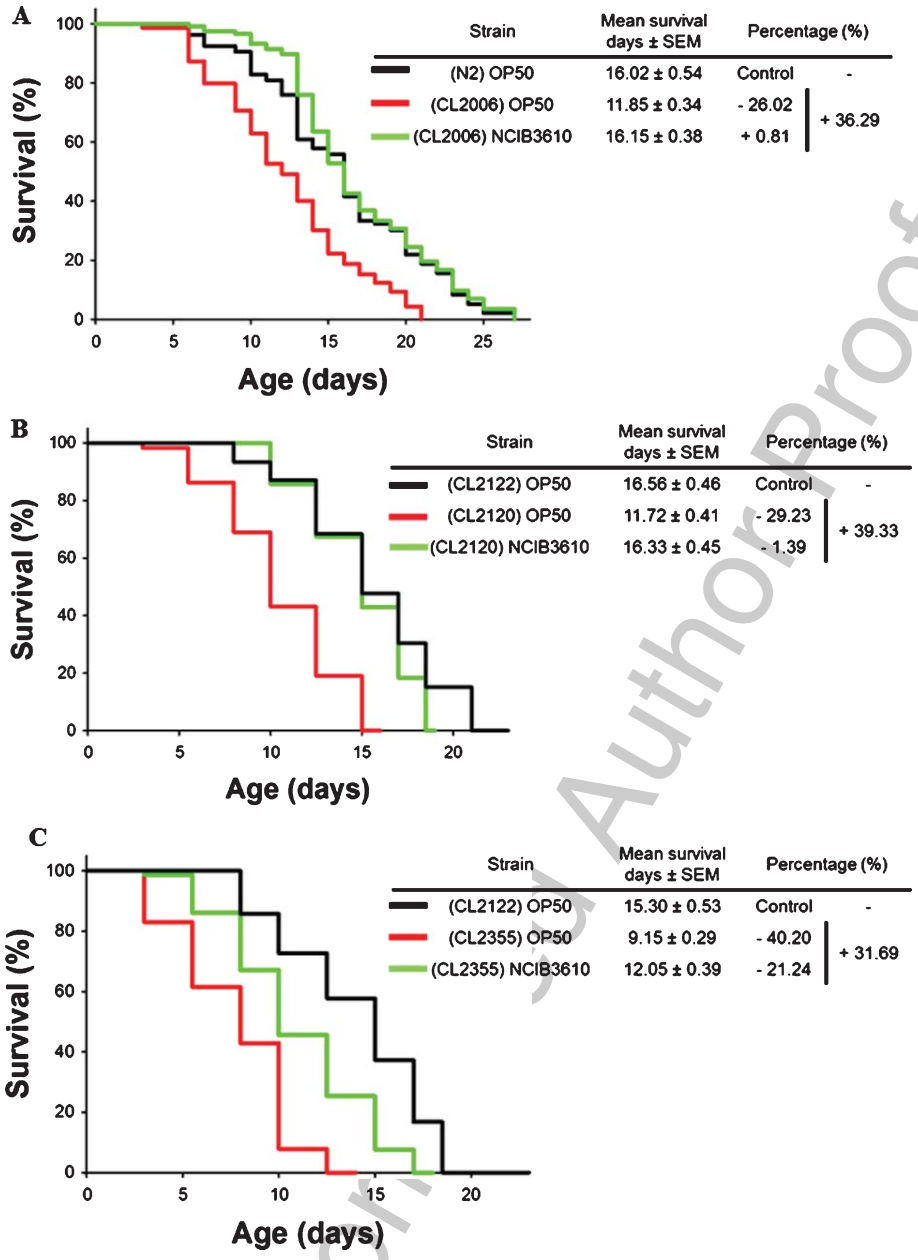


Fig. 6. Probiotic *B. subtilis* produced a healthy lifespan for Aβ-synthesizing *C. elegans*. Lifespan of OP50- and NCIB3610-colonized AD model CL2006 (A), CL2120 (B), and CL2355 (C) worms. Worms were grown on bacteria-seeded 10-cm NGM agar plates at 20 or 23°C (for A-B and C, respectively), and survival was monitored as indicated until the last worm died (see Materials and Methods for details). Control wild-type worm strains are N2 and Cl2122 for A and B-C, respectively. A typical output of three independent experiments performed in duplicate is presented.

[63, 64]. Dietary restriction, a condition of reduced caloric intake [65], enhances longevity and protects against proteotoxicity by a mechanism (distinct from reduced IILS signaling) that requires HSF-1 activation [66]. Both longevity routes regulate genes (either repressing or activating them in the case of IILS or DR, respectively) involved in protection against oxidative stress, inflammation, microbial infections, and the production of numerous proteins with chaperone activity to maintain the integrity of protein homeostasis against proteotoxicity [20, 21, 63, 66]. *Bacillus subtilis* has a prolongevity (anti-aging) effect

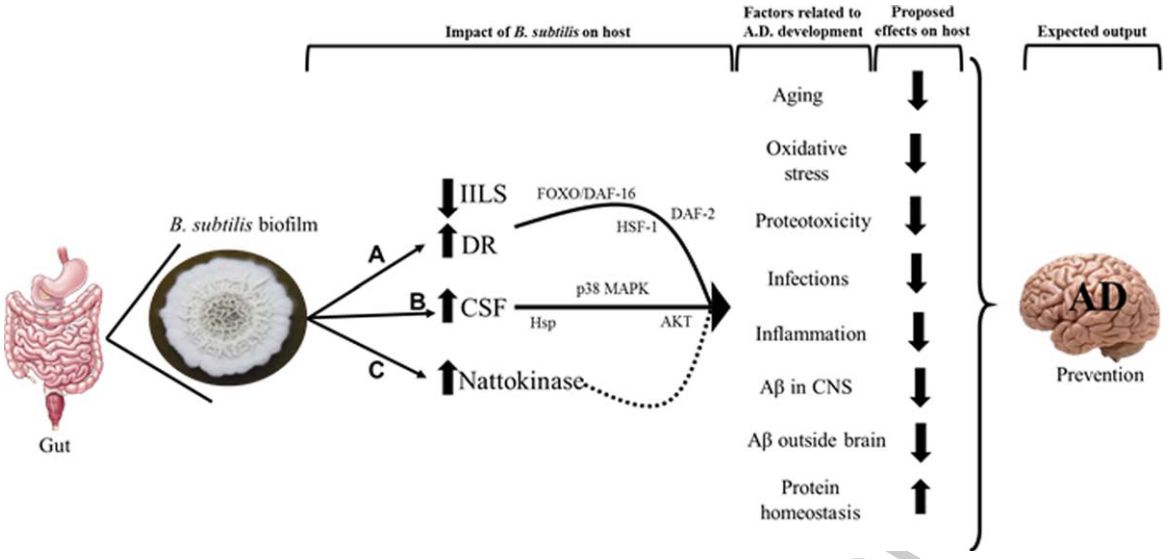
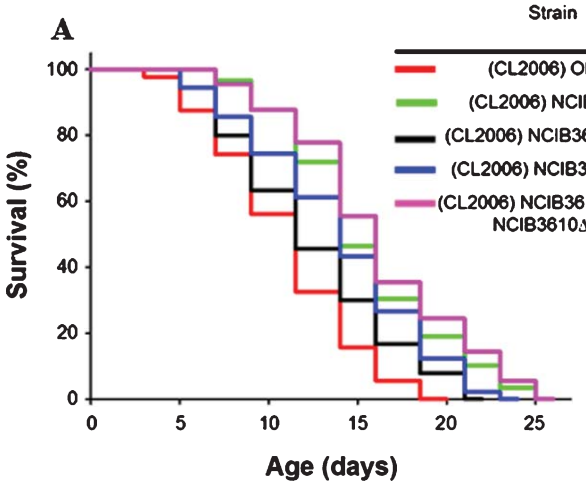


Fig. 7. A workable proposed model for the *B. subtilis* effects in AD. Cartoon that summarizes the different routes (anti-aging ILS downregulation/DR upregulation, A; CSF quorum-sensing peptide production, B; and Aβ-degrading nattokinase activity, C) that probiotic *B. subtilis* might use to produce beneficial effects against factors related to AD development and, therefore, in fighting against human AD (see Discussion for details).

because, when colonizing the host intestine through the formation of a beneficial gut-associate biofilm, it downregulates and upregulates the IILS pathway and the process of dietary restriction (DR), respectively [19, 39]. The anti-aging effect of *B. subtilis* occurred in 90% because to ILS inhibition and in 10% due to DR activation [19, 39]. We envision that the prolongevity effect of *B. subtilis* would protect against the aging-linked risk factors that are associated with AD development (Fig. 7A).

The second pathway that *B. subtilis* would use to delay or fight AD is through the production of the quorum-sensing (QS) pentapeptide CSF (also named PhrC) [19, 67]. QS is a chemical mechanism that bacteria use for cell-to-cell communication with other bacteria (inrtra-) or plants and animals (inter-specific kingdom communication [68]. Basically, bacteria produce small metabolites (i.e., acyl-homoserine lactones and short peptides), QS molecules, which are liberated to the surrounding environment wherein other organisms detect and internalize them. Once inside the host cells, the bacterial QS molecules affect host gene expression. The CSF pentapeptide plays a crucial intra-specific role in orchestrating cell-to-cell communication in vital *B. subtilis* lifestyle processes such as natural DNA competence, sporulation, and biofilm formation [67]. Besides this intraspecific (bacterium-bacterium interaction) role of CSF, there is a reported interspecific CSF function (bacterium-mammalian inter-kingdom interaction) [40, 69]. This quorum-sensing pentapeptide is internalized via the mammalian oligopeptide transporter OCTN2, where it induces the production of the heat shock protein chaperone Hsp27 and the p38 MAPK and AKT survival pathways [69]. This induction leads to cellular protection against oxidative stress, misfolded proteins, and loss of barrier function [69]. *In vitro*, Hsp27 acts as an ATP-independent chaperone by inhibiting protein aggregation and stabilizing misfolded proteins, actions that ensure refolding by the Hsp70 complex [70]. The Hsp27 also activates the proteasome complex to quicken the degradation of irreversibly denatured or aberrant proteins [71, 72]. Diverse proteomic analysis showed that there is a complex map of protein alterations in AD; these findings indicate that AD is more than an Aβopathy or tauopathy: it is a proteopathy [36, 72–74]. In this sense, the expression of protective HSPs (i.e., Hsp27 and other chaperons) and survival pathways (MAPK and AKT) induced by probiotic quorum sensing (i.e., CSF) might keep Aβ oligomers, and other aberrant proteins related to AD, at sub-toxic concentrations in the brain and other body sites (Fig. 7B).

To obtain experimental support for the proposed roles of both *B. subtilis* properties as novel anti-AD weapons, we performed lifespan assays in *B. subtilis* isogenic NCIB3610 mutant strains affected by the prolongevity effect and CSF production (Fig. 8).



Strain	Mean survival days ± SEM	Percentage (%) with respect to control	Percentage (%) with respect to control
(CL2006) OP50	12.01 ± 0.42	Control	-
(CL2006) NCIB3610	16.53 ± 0.47	+ 37.63	Control
(CL2006) NCIB3610Δ*bslA*	13.56 ± 0.48	+ 12.90	- 17.97
(CL2006) NCIB3610Δ*csf*	15.01 ± 0.51	+ 24.98	- 9.19
(CL2006) NCIB3610Δ*bslA* + NCIB3610Δ*csf*	17.17 ± 0.50	+ 42.96	+ 3.87

Fig. 8. Role of the anti-aging effect and CSF production for the anti-AD effect of probiotic *B. subtilis*. Lifespans of the AD CL2006 *C. elegans* strain colonized by different bacterial strains: OP50, wild-type NCIB3610 and isogenic NCIB3610 strains deficient in biofilm formation (Δ*bslA*) or CSF production (Δ*csf*). Worms were grown on bacteria-seeded 10-cm NGM agar plates at 20°C, and survival was monitored as indicated until the last worm died (see Materials and Methods for details). A typical output of three independent experiments performed in duplicate is presented.

Most of the *B. subtilis* prolongevity effect is mediated by the proficiency of *B. subtilis* to form a healthy biofilm in the host gut [19, 75]. Therefore, we used an isogenic NCIB3610 Δ*bslA* mutant strain deficient in biofilm formation [19, 75] to test the contribution of the anti-aging effect of the bacterium to the protection against human Aβ peptide expression. As shown in Fig. 8, the mean lifespan of CL2006 AD worms colonized by NCIB3610-Δ*bslA* cells decreased by 18% compared with CL2006 worms colonized by wild-type NCIB3610 cells ($n = 100$, $p < 0.001$). Similarly, CL2006 worms colonized by NCIB3610-Δ*csf* cells (deficient in CSF synthesis) displayed a shorter lifespan (~9% decrease of mean lifespan) compared with CL2006 worms colonized by wild-type NCIB3610 cells ($n = 100$, $p < 0.001$; Fig. 8). Because BslA and CSF are secreted to the extracellular matrix of the biofilm [19], mixtures of Δ*bslA* and Δ*csf* mutant cells complement each other to restore full biofilm-formation and CSF-production proficiencies [19]. Therefore, we fed CL2006 worms on a 50:50 mixture of Δ*bslA* and Δ*csf B. subtilis* cells and measured the lifespan effect produced by this mixture. As shown in Fig. 8, the colonization of CL2006 worms by a mixture of both *B. subtilis* mutant strains (Δ*bslA* and Δ*csf* cells in equal proportions) restored the lifespan to a level indistinguishable of the lifespan of CL2006 worms colonized by wild-type NCIB3610 cells (mean values of 17.17 ± 0.50 days and 16.53 ± 047 days, respectively, $n = 100$, $p < 0.001$; Fig. 8). These results support the novel roles of the anti-aging effect and CSF-synthesis proficiencies of *B. subtilis* against AD (Fig. 7A, B).

There is a third pathway that *B. subtilis* might use against AD, that is the production of nattokinase, a 27.7-kDa serine enzyme produced by this bacterium [76, 77]. This protease is found in the Japanese fermented food natto [76–78], and nattokinase-like proteases are probably also found in other Asian and African functional fermented foods [79]. Nattokinase gained tremendous popularity as a fibrin-degrading and clot-dissolving agent [76–78]. Interestingly, *in vivo*, nattokinase can be absorbed across the human intestinal tract [80, 81], and *in vitro*, it can degrade Aβ oligomers [82, 83]; Aβ oligomers ($A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$) are formed and deposited in the CNS as well as in intestinal epithelial cells and the enteric nervous system [84–87]. Therefore, intestinally produced nattokinase (and CSF) would help decrease Aβ oligomers in the gastrointestinal tract [88, 89]. This phenomenon is important because intestinal Aβ oligomers would interact with immune cells and enteric neurons to be (at least partly) responsible for the gastrointestinal dysfunctions of elderly, including AD patients. Moreover, intestinal Aβ co-localizes with the lipoprotein ApoB, and in wild-type mice (fed on a diet rich in saturated fatty acids), deposits of both proteins were found in the brain [60]. These data suggest that ApoB-Aβ complexes produced in the intestine might deliver intestinal-produced Aβ to the

brain [60]. Alternatively, intestinally absorbed nattokinase (and/or CSF) might use the gut-brain-axis [60] to reach the leaky blood-brain barrier of elderly and AD patients to exert their beneficial effects *in situ* in the CNS [9, 60, 91, 92]. Under these different scenarios, the beneficial role of nattokinase in the *in vivo* degradation of Aβ oligomers deserves future investigations (Fig. 7C).

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