Lactobacilli in a clade ameliorate age-dependent decline of thermotaxis behavior in *Caenorhabditis elegans*

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Abstract

Diet is proposed to affect brain aging. However, the causality and mechanism of dietary effects on brain aging are still unclear due to the long time scales of aging. The nematode *Caenorhabditis elegans* (*C. elegans*) has led aging research because of its short lifespan and easy genetic manipulation. When fed the standard laboratory diet, *Escherichia coli* (*E. coli*), *C. elegans* experiences an age-dependent decline in temperature-food associative learning, called thermotaxis. To address if diet ameliorates this decline, we screened 35 different lactic acid bacteria as alternative diets. We found that *Lactobacilli* in a clade enriched with heterofermentative bacteria ameliorated age-dependent decline. On the other hand, homofermentative *Lactobacillus* species did not show this beneficial effect. *Lactobacilli* affected the thermotaxis of aged animals through DAF-16, an ortholog of mammalian FOXO transcription factor, while the effect on the thermotaxis was independent of the lifespan and locomotion. Our results demonstrate that diet can impact brain aging without changing the lifespan and that bacterial screen using *C. elegans* is a powerful approach to investigate age-dependent behavioral decline.

Introduction

Human life expectancy has increased since the nineteenth century (Dong, Milholland, & Vijg, 2016), which has led to the social problem related to age-dependent cognitive dysfunction. Although human studies suggest that genetic background, diet, and lifestyle might affect brain aging, the possible mechanism of how they affect brain aging remain unclear (Deary et al., 2009).

The nematode *Caenorhabditis elegans* (*C. elegans*) is ideal for addressing the mechanism of age-related phenotypes because of the two to three-week lifespan and the variety of available genetic tools. In *C. elegans*, the effect on age-related phenotypes can be readily separable from the organismal lifespan by directly measuring the lifespan. In the past decades, studies using *C. elegans* have led aging research by revealing the mechanism of how dietary restriction, insulin-like signaling, and germline stem cells affect organismal lifespan (Mack, Heimbucher, & Murphy, 2018; Wolff & Dillin, 2006). Like mammals, *C. elegans* experiences age-dependent functional changes in the nervous system (Stein & Murphy, 2012). Aged worms are defective in locomotion (Mulcahy, Holden-Dye, & O'Connor, 2013), mechanosensory response (Beck & Rankin, 1993), chemotaxis (Leinwand et al., 2015), thermotaxis (Huang et al., 2020; H. Murakami, Bessinger, Hellmann, & Murakami, 2005; S. Murakami & Murakami, 2005), and food-butanone associative learning (Kauffman, Ashraf, Corces-Zimmerman, Landis, & Murphy, 2010). Emerging evidence suggests that genetic manipulations can prevent age-dependent functional decline in the *C. elegans* nervous system. The mutation of kynurenic acid synthesizing enzyme *nkat-1* prevents age-dependent memory decline in the food-butanone association (Vohra, Lemieux, Lin, & Ashrafi, 2018). Overactivation of Gα signaling in AWC sensory neurons also maintains the ability to form memory in aged worms in the food-butanone association (Arey, Stein, Kaletsky, Kauffman, & Murphy, 2018).

The modification of diet can be easily applicable to our daily lives, compared to genetic manipulations. Studies in humans and mice imply that diets affect the cognitive decline in aged animals (Joseph, Cole, Head, & Ingram, 2009; Vauzour et al., 2017). Here, we use *C. elegans* to address the dietary effect on the age-dependent decline in behavior and its underlying mechanism. In laboratories, *C. elegans* is maintained monoxenically with a uracil auxotroph *Escherichia coli* (*E. coli*) strain, OP50, as

the standard diet (Brenner, 1974). On the other hand, *C. elegans* in natural habitat eats a wide variety of bacteria (Berg et al., 2016; Dirksen et al., 2016; Johnke, Dirksen, & Schulenburg, 2020; Samuel, Rowedder, Braendle, Felix, & Ruvkun, 2016; Zhang et al., 2017). These bacteria affect the physiology of *C. elegans*, such as growth rate, reproduction, and sensory behavior (Dirksen et al., 2016; O'Donnell, Fox, Chao, Schroeder, & Sengupta, 2020; Samuel et al., 2016). However, the effect of different bacteria on brain aging is unexplored. Among the potential bacterial diet for *C. elegans* in natural habitat (Berg et al., 2016; Dirksen et al., 2016; Samuel et al., 2016), we focused on Lactic Acid Bacteria (LAB), which are the most commonly used probiotics for humans (Hill et al., 2014). LAB, such as *Lactobacilli* (*Lb.*) and *Bifidobacteria* (*B.*), are gram-positive, non-spore-forming bacteria that produce lactic acid from carbohydrates as the primary metabolic product. Depending on the species, LAB have various effects on *C. elegans* physiology. *Lb. gaseri*, *B. longum*, and *B. infantis* extend lifespan in *C. elegans* (Komura, Ikeda, Yasui, Saeki, & Nishikawa, 2013; Nakagawa et al., 2016; L. Zhao et al., 2017). On the other hand, *Lb. helveticus* does not increase the lifespan (Nakagawa et al., 2016). Even in the same species, different strains have different effects on the lifespan, body size, and locomotion (Wang et al., 2020). In *C. elegans*, LAB modulate evolutionarily conserved genetic pathways such as insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway (Grompone et al., 2012; Sugawara & Sakamoto, 2018), which consists of insulin receptor DAF-2, phosphoinositide 3 (PI3) kinase cascade, and downstream transcription factor DAF-16 (Lin, Dorman, Rodan, & Kenyon, 1997). DAF-16 is a sole *C. elegans* ortholog of mammalian FOXO transcription factor and involved in multiple biological processes (Stein & Murphy, 2012; Tissenbaum, 2018).

To comprehensively understand the effect of LAB, we screened 35 different LAB species, including some subspecies (Table S1). We examined the age-dependent functional decline of thermotaxis behavior, which reflects associative learning between temperature and food (Hedgecock & Russell, 1975; Mori & Ohshima, 1995). We demonstrate that *Lactobacilli* in a clade prevent the age-dependent decline in thermotaxis behavior in *C. elegans*. Among beneficial LAB, we demonstrated that the beneficial effect of *Lb. reuteri* is dependent on *daf-16*, but independent of the effect on lifespan and locomotion.

Results

C. elegans thermotaxis behavior declines with age

When cultured with food at a temperature within the physiological range (15~25 °C), *C. elegans* migrates toward and stays at the cultivation temperature (T_{cult}) on a linear thermal gradient without food (Fig. 1A). This behavior is called thermotaxis (Hedgecock & Russell, 1975; Mori & Ohshima, 1995). To see the effect of the standard diet on thermotaxis at different ages, we cultivated worms at 20 °C with an *E. coli* strain, OP50 (hereafter, *E. coli*), which is the most commonly used diet in the laboratory condition (Brenner, 1974). When the worms were placed at 17 °C on a temperature gradient without food, young adults (day 1 of adulthood, D1) migrated up the temperature gradient toward 20 °C (Fig. 1B). On the other hand, aged worms (day 5 of adulthood, D5) remained around the spotted area and did not reach the area near T_{cult} (Fig. 1B), as previously reported (Huang et al., 2020). To evaluate the ability to perform the thermotaxis behavior, we defined the performance index, which indicates the fraction of worms around T_{cult} (Fig. 1A). The performance index declined from D1 to D5. This low performance is not due to an inability to move because D5 worms cultivated at 20 °C could migrate down the thermal gradient relatively normally when the origin was at 23 °C (Fig. 1B, also see below). To further accelerate aging by cultivating at high temperature (Klass, 1977), we cultured worms at 23 °C and placed them on a temperature gradient centered at 20 °C (Fig. S1). In this condition, worms gradually lost the ability to move toward T_{cult} (Fig. S2), and the performance index declined during aging from ~0.75 at D1 to ~0.25 at D5 (Fig. 1D). Therefore, we determined to use the thermotaxis behavior of D5 worms cultivated at 23 °C to analyze dietary effects on brain aging.

Specific LAB prevent the aged-dependent decline of thermotaxis behavior

To address if diet affects the age-dependent decline in thermotaxis behavior, we fed worms with different LAB species instead of the regular *E. coli* diet. We selected 35 LAB, consisting of 17 *Lactobacilli (Lb.)*, two *Pediococci (P.)*, two *Lactococci (Lc.)*, two *Streptococci (S.)*, five *Leuconostoc (Ls.)*, and seven *Bifidobacteria (B.)* (Table S1). To avoid developmental effects by feeding with LAB, we fed worms with

114 *E. coli* until D1 and fed LAB from D1 to D5 (Fig. 2A). Worms were cultivated at 23 °C and spotted at
115 20 °C on the thermal gradient for thermotaxis assays (Fig. 2A). Five LAB did not support the survival of
116 worms during aging (Fig. 2B, NA). Compared to *E. coli*, 22 LAB significantly increased the performance
117 indices of the aged worms, while eight LAB did not affect them (Fig. 2B). Aged worms fed *P.*
118 *pentosaceus*, *Lb. reuteri*, *Lb. rhamnosus*, and *Lb. plantarum* showed the highest performance indices,
119 which are not lower than D1 adults (Fig. 2B). With the temperature gradient of 17-23 °C, aged worms fed
120 these four LAB migrated to the T_{cult}, while *E. coli*-fed aged worms distributed around the spotted area
121 (Fig. S3A).

122 We first ruled out the possibility that the constitutive thermophilicity caused apparent high
123 performance indices of LAB-fed worms, irrespective of the association between food and temperature.
124 Thermophilicity is reported for mutants of genes such as *pkc-1*/*ttx-4* encoding protein kinase C (Okochi,
125 Kimura, Ohta, & Mori, 2005) and *tax-6* encoding calcineurin A subunit (Kuhara, Inada, Katsura, & Mori,
126 2002). To distinguish between associative learning and thermophilicity, we shifted the thermal gradient of
127 the assay plate from 17-23 °C to 20-26 °C. As previously reported, *tax-6* mutants migrated toward higher
128 temperature than T_{cult} (Fig. S3A). On the other hand, LAB-fed D5 worms crawled around the T_{cult} instead
129 of migrating toward higher temperature (Fig. S3A). To quantitate the thermal preference of worms, we
130 calculated the thermotaxis index instead of the performance index (Ito, Inada, & Mori, 2006) (Fig. 1A).
131 Unlike thermophilic *tax-6* mutants, LAB-fed D5 worms did not show higher thermotaxis indices than D1
132 wild type worms (Fig. S3B), suggesting that LAB-fed D5 worms were not constitutively thermophilic.

133 We next addressed if LAB-fed D5 worms can remember a new temperature by shifting the T_{cult}
134 from 23 °C to 17 °C one day before the thermotaxis assay. D1 animals could reset the temperature
135 memory and migrate toward the new T_{cult}, 17 °C (Fig. 3). Compared to D1 worms, *E. coli*-fed D5 worms
136 responded to the temperature shift marginally (Fig. 3). On the other hand, LAB-fed D5 worms showed a
137 similar behavioral change to D1 worms (Fig. 3). This result suggests that LAB-fed aged worms retained
138 the ability to remember the new T_{cult}.

C. elegans shows preference in the bacterial diet (Shtonda & Avery, 2006). In our LAB screen (Fig. 2B), we used different foods, namely *E. coli* and LAB, during temperature-food association. It raised the possibility that LAB ameliorated the performance decline by serving as better food than *E. coli*. To examine this possibility, we switched the diet between *E. coli* and LAB one day before the thermotaxis assay (Fig. 4A). We used *Lb. reuteri* because it was one of the top hits in our screen and did not affect the organismal lifespan, as mentioned below. Aged worms whose diet was switched from *Lb. reuteri* to *E. coli* showed the high performance index, while aged worms with the opposite condition did not. This result ruled out the possibility that the better performance index of *Lb. reuteri*-fed worms was due to a stronger association between *Lb. reuteri* and temperature and suggests that aged worms can sense *E. coli* normally and associate it with temperature when fed *Lb. reuteri* during aging.

To directly test if *E. coli*-fed aged worms can sense food, we performed a food recognition assay of aged worms (Sawin, Ranganathan, & Horvitz, 2000). Well-fed young worms slowed their body bends on the plate with *E. coli* (basal slowing response), and even more so when starved young worms were placed on the plate with *E. coli* (enhanced slowing response) as previously reported (Fig. 4B) (Sawin et al., 2000). We found that *E. coli*-fed aged worms also showed normal basal response (Fig. 4C). The food sensation of aged worms is also reported to be normal using chemotaxis assay with *E. coli* (Cornils et al., 2016). These results suggest that the low performance in the thermotaxis of aged worms is not due to the defective sensation of *E. coli* as food. In contrast to the basal slowing response, the enhanced slowing response in aged worms was not statistically significant (Fig. 4C), implying that aged worms might not sense starvation normally.

Altogether, we concluded that the high performance indices of the aged worms fed some lactic acid bacteria were not due to the thermophilicity or the different association of the food, but due to better thermotaxis ability of the aged worms.

Effects of LAB on lifespan and locomotion vary

Some LAB extend the lifespan of *C. elegans* (Komura et al., 2013; Nakagawa et al., 2016; Wang et al., 2020; Y. Zhao et al., 2013). The apparent better performance of LAB-fed aged worms in thermotaxis might be a consequence of the systemic effects of prolonged organismal lifespan. To test this possibility, we measured the lifespan of worms fed LAB from D1 which had beneficial effects on thermotaxis of aged worms (Figs. 2B and 5A): *P. pentosaceus*, *Lb. reuteri*, *Lb. rhamnosus*, and *Lb. plantarum.* We measured the lifespan of worms fed LAB. To avoid the growth of *E. coli* on LAB plates after transferring worms, we used peptone-free NGM plates (T. Ikeda, Yasui, Hoshino, Arikawa, & Nishikawa, 2007; Lee, Kwon, & Lim, 2015) (see Materials and Methods for details). Peptone-free plates did not affect the beneficial effects of *Lb. reuteri* compared to *E. coli* in thermotaxis of aged worms (Fig. S4). LAB had various effects on the lifespan of worms: *P. pentosaceus* prolonged the lifespan; *Lb. reuteri* did not affect the lifespan; *Lb. rhamnosus* and *Lb. plantarum* shortened the lifespan (Fig. 5A). This result suggests that the beneficial effect of LAB on thermotaxis is not due to prolonged lifespan at least for three among four selected LAB.

We next asked if the beneficial LAB on thermotaxis ameliorated other age-dependent behavioral decline and assessed the locomotion of aged worms fed selected LAB using two assays: thrashing assay (Miller et al., 1996) and motility on plates with food. As previously reported (Mulcahy et al., 2013), aged worms fed *E. coli* showed locomotion defects in both assays (Figs. 5B and 5C). In the thrashing assay, *Lb. reuteri*- and *Lb. rhamnosus*-fed aged worms showed better locomotion than *E. coli*-fed aged worms, while *P. pentosaseus* and *Lb. plantarum* did not have effects (Fig. 5B). In the motility assay, *Lb. plantarum*- and *Lb. rhamnosus*-fed aged worms showed reduced locomotion than *E. coli*-fed aged worms, while *P. pentosaseus* and *Lb. reuteri* did not have effects (Fig. 5C). Thus, the effects of four *Lactobacilli* on the two locomotion assays varied, although these bacteria had similar effects on thermotaxis, suggesting that LAB might have different effects on different types of neurons.

188 **Beneficial LAB are enriched in a clade of *Lactobacilli***

189 To understand the common feature of LAB strains that ameliorated aged-dependent thermotaxis decline
190 in our screen, we first observed the morphologies of four top hit strains (Fig. S5). However, their
191 morphologies and sizes are different from each other; these physical properties of LAB may not explain
192 high performance indices of aged worms. We next made a phylogenetic tree of 35 LAB strains with the
193 heatmap of the associated thermotaxis performance indices (Fig. 6A). This analysis revealed that LAB
194 associated with high performance indices were significantly enriched in a specific clade henceforth
195 referred to as Clade A (Figs. 6A and 6B). This clade containing *Lactobacilli* and *Pediococci* is enriched in
196 obligatory and facultatively heterofermentative species except for *P. pentosaceus*, which is obligatory
197 homofermentative (Fig. 6B). On the other hand, the *Lactobacilli*, which are associated with relatively low
198 thermotaxis indices (referred to as Clade B), are all homofermentative (Fig. 6B).

199 To get an insight into whether LAB affect worms as live bacteria or serve as nutrition, we
200 examined the effect of bacteria heat-killed at 65 °C for one hour (Fig. 6C, see Materials and Methods).
201 Like aged worms fed live bacteria, ones fed heat-killed *E. coli* and heat-killed *Lb. reuteri* showed low and
202 high performance indices in thermotaxis, respectively. This result suggests that the effect of bacteria on
203 thermotaxis is independent of the condition of being alive.

204

205 ***daf-16* is involved in the beneficial effect of *Lb. reuteri* in thermotaxis of aged worms**

206 We addressed the molecular mechanism of how worms respond to the LAB diet. LAB can induce dietary
207 restriction, which leads to a prolonged lifespan (Y. Zhao et al., 2013). *pha-4*, an ortholog of the human
208 FOXA2 transcription factor, is required for dietary restriction-induced longevity, and its expression is
209 increased by dietary restriction (Panowski, Wolff, Aguilaniu, Durieux, & Dillin, 2007). In our condition,
210 *pha-4* expression decreased in LAB-fed aged worms compared to *E. coli*-fed aged worms (Fig. 7A).
211 Moreover, *eat-2* mutants, which show dietary restriction by defective pharyngeal pumping, did not
212 increase the performance index of *E. coli*-fed aged worms in thermotaxis (Fig. 7B). These results suggest

that dietary restriction on its own does not increase thermotaxis performance in aged worms and that good thermotaxis performance of LAB-fed aged worms is likely independent from dietary restriction.

We next tested several mutants that might be responsible for the beneficial effect of *Lb. reuteri* in thermotaxis when aged. *nkat-1* and *kmo-1* genes that encode enzymes in the kynurenic acid synthesizing pathway are known to be involved in butanone-associated memory in aged animals (Vohra et al., 2018); *daf-16* is an ortholog of mammalian FOXO transcription factor involved in longevity (Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993) and LAB-dependent lifespan extension (Grompone et al., 2012; Lee et al., 2015; Sugawara & Sakamoto, 2018). Aged *nkat-1* and *kmo-1* mutants maintained thermotaxis ability like wild type when fed *Lb. reuteri*. On the other hand, aged *daf-16* mutants showed significantly less ability to perform thermotaxis than its D1 counterpart (Fig. 7C); aged *daf-16* mutants fed *Lb. reuteri* distributed around a temperature slightly lower than the T_{cult} (Fig. 7D). This decreased thermotaxis ability in aged *daf-16* mutants fed *Lb. reuteri* was not due to shortened lifespan because *daf-16* mutants had comparable lifespan to wild type animals when fed *Lb. reuteri* (Fig. 7E). Collectively, our data implies that *daf-16* is involved in the effect of *Lb. reuteri* on thermotaxis in aged animals.

Discussion

The causal relationship between diets and their effects on animals' physiology is challenging to address in humans or mammalian models because microbiota in the gut and diet are complex. It is especially true in the context of aging because of their long lifespan. Using *C. elegans* as a model, we clearly showed evidence of the dietary effect on the age-dependent behavioral decline discernible from the lifespan.

LAB effects on aging vary among different phenotypes

In this study, we demonstrated that LAB affect the age-dependent decline of associative learning by ruling out the possibilities that the apparent high performance indices of LAB-fed aged worms were due to thermophilicity, stronger association to LAB, better motility, or longer lifespan. The major thermosensory neuron AFD (Mori & Ohshima, 1995) can store temperature memory even when isolated (Kobayashi et al., 2016). Although Ca^{2+} response in AFD is reported to be defective in aged worms (Huang et al., 2020), the temperature sensation itself does not seem to be abolished in aged worms because they could migrate down the gradient. AFD thermosensory neurons synapse onto and regulate AIY interneurons by switching excitatory and inhibitory signals in a context-dependent manner (Mori & Ohshima, 1995; Nakano et al., 2020; White, Southgate, Thomson, & Brenner, 1986). AIY neurons are reported to be a cite of action of *age-1* PI3 kinase, which is upstream of *daf-16* in isothermal tracking behavior (H. Murakami et al., 2005). Given that the beneficial effect of *Lb. reuteri* on thermotaxis of aged worms is *daf-16*-dependent, *E. coli*-fed aged worms might have defects in AIY interneurons.

We found that *E. coli*-fed worms declined the ability to perform thermotaxis during aging more severely when subjected to migrate up the thermal gradient than when subjected to migrate down the gradient (Figs. 1B and 1C). Thermotaxis behavior is achieved by multiple steps: sensing temperature, recognizing food, associating food and temperature, memorizing T_{cult}, and migrating toward T_{cult} (Aoki & Mori, 2015; Goodman & Sengupta, 2018; Kimata, Sasakura, Ohnishi, Nishio, & Mori, 2012). Thus, the different severities of thermotaxis decline between migration up and down the gradient in aged animals might be attributed to the different neural circuits responsible for migrating up and down the thermal

gradient as reported previously (M. Ikeda et al., 2020). Despite the similar beneficial effects of *P. pentosaceus, Lb. reuteri, Lb plantarum,* and *Lb. rhamnosus* on thermotaxis in aged worms, these LAB showed various effects on locomotion, suggesting that the effects of aging vary depending on the neurons. Even in the same neurons, the functional aging depends on the context (Leinwand et al., 2015).

Neuronal aging is also discernible from an organismal lifespan. *nkat-1* mutants prevent age-dependent memory decline in associative learning between food and butanone without changing lifespan (Vohra et al., 2018). Similarly, we found that *Lb. reuteri* improved thermotaxis in aged worms without changing the lifespan. More strikingly, *Lb plantarum* and *Lb. rhamnosus* shortened the lifespan while they had beneficial effects on the thermotaxis of D5 adults. This different dietary condition allows us to address the mechanism underlying phenotypic variation in aged animals independent from organismal lifespan and genetic perturbation.

How do the LAB affect the age-dependent decline in thermotaxis?

Previous reports elucidated how bacterial diet affects *C. elegans* as nutritional components, gut microbiota, or pathogen (Kumar et al., 2019; J. J. Zhou, Chun, & Liu, 2019). Bacterial diet can change *C. elegans* metabolites (Gao et al., 2017; Reinke, Hu, Sykes, & Lemire, 2010) and gene expression (MacNeil, Watson, Arda, Zhu, & Walhout, 2013).

Both live *E. coli* and LAB can colonize in worms (Berg et al., 2016; Chelliah et al., 2018; Park et al., 2018; Portal-Celhay, Bradley, & Blaser, 2012). Live bacteria are necessary for some physiological roles; secreted enterobactin from live *E. coli* in the gut promotes *C. elegans* growth (Qi & Han, 2018); live, but not dead, LAB reduced the susceptibility to pathogenic bacteria *Pseudomonas aeruginosa*. On the other hand, live bacteria are unnecessary in different contexts; heat-killed *Lb. paracasei* and *Bifidobacterium longum* extend *C. elegans* lifespan (Sugawara & Sakamoto, 2018; Wang et al., 2020). In our thermotaxis assay on aged worms, *E. coli* and LAB killed by 65 °C treatment had similar effects to live bacteria. This result implies that, instead of live bacteria, heat-resistant metabolites might be responsible for the effect on thermotaxis of aged *C. elegans*. Metabolites in bacterial diet affect *C.*

279 *elegans* physiology; some metabolites are beneficial, while others are toxic (J. J. Zhou et al., 2019).
280 Coenzyme Q in *E. coli* shortens the lifespan of *C. elegans* (Larsen & Clarke, 2002). Bacterial nitric oxide
281 and folate also positively and negatively regulate *C. elegans* lifespan, respectively (Gusarov et al., 2013;
282 Virk et al., 2012). Vitamin 12 in *Comamonas aquatica* accelerates development and reduces fertility
283 without changing lifespan (Watson et al., 2014). Given that different metabolites are produced by
284 different LAB (Tomita, Saito, Nakamura, Sekiyama, & Kikuchi, 2017), these metabolites might be
285 responsible for the different effects on the thermotaxis of aged *C. elegans*.
286 Our results indicated that LAB associated with high performance indices of thermotaxis are
287 associated with a clade enriched in heterofermentative *Lactobacilli* and *Pediococci* (Clade A in Figure
288 5A). Heterofermentative LAB produce not only lactic acid and ATP but also several other end products
289 such as ethanol and CO_2 from glucose. On the other hand, homofermentative LAB convert glucose into
290 two molecules of lactic acid and ATP. Heterolactic fermentation itself does not explain high performance
291 index in thermotaxis of aged worms because heterofermentative *Leuconostoc* and *Bifidobacteria* species
292 did not give high performance indices. Metabolites other than lactic acid, ethanol, and CO_2 are also
293 different between hetero- and homofermentative *Lactobacilli* (Tomita et al., 2017). Metabolites enriched
294 in heterofermentative *Lactobacilli* include a neurotransmitter GABA and tyramine, a substrate to
295 synthesize neurotransmitter octopamine. We note that Tomita *et al*. reported the metabolites in the media
296 (Tomita et al., 2017) while we supply bacteria to worms after washing off the bacterial media.
297 Nonetheless, metabolites enriched in heterofermentative *Lactobacilli* are possibly beneficial effects on the
298 age-dependent decline in thermotaxis.
299
300 **Diets modulate genetic pathways in *C. elegans***
301 LAB can extend the lifespan of *C. elegans* either by dietary restriction-dependent (Y. Zhao et al., 2013) or
302 by dietary restriction-independent mechanisms (Komura et al., 2013; Nakagawa et al., 2016). The
303 mechanism underlying the beneficial effect on the thermotaxis of aged worms does not seem to depend on
304 the activation of the dietary restriction pathway by the LAB. First, the expression of *pha-4* was low.

Second, the lifespan of LAB-fed worms was not necessarily prolonged. Third, *eat-2* mutants, which mimic dietary restriction, did not improve thermotaxis in aged worms fed *E. coli*. Fourth, *kmo-1* and *nkat-1* genes, which are involved in dietary restriction-dependent beneficial effects on associative learning (Vohra, Lemieux, Lin, & Ashrafi, 2017), did not affect the dietary effects on thermotaxis of aged worms.

Different LAB activate distinct genetic pathways such as insulin and IGF-1 signaling (IIS) pathway important for lifespan regulation and p38 mitogen-activated protein kinase (MAPK) pathway important for innate immunity. *Lb. rhamnosus* and *B. longum* extend the lifespan of *C. elegans* by modulating the IIS pathway consisting of DAF-2 and DAF-16 (Grompone et al., 2012; Sugawara & Sakamoto, 2018). *B. infantis* extends the lifespan of *C. elegans* via the PMK-1 p38 MAPK pathway and a downstream transcription factor SKN-1, an ortholog of mammalian Nrf, but not via DAF-16 (Komura et al., 2013). The PMK-1 pathway is also activated by *Lb. acidophilus* and *Lactobacillus fermentum* (Kim & Mylonakis, 2012; Park et al., 2018). Worms fed a lactic acid bacteria, *Weissella*, show higher expression of *daf-16*, *aak-2*, and *jnk-1*, and extend lifespan in these genes-dependent manners (Lee et al., 2015). In our results, *daf-16* was dispensable in thermotaxis at D1, but necessary for beneficial effects of *Lb. reuteri* at D5, suggesting a specific role in the LAB's effects on aged worms. Given that *daf-16* has neuron-specific targets (Kaletsky et al., 2016), differential expressions of these genes with different diets can affect thermotaxis behavior. As discussed above, *daf-16* might function in AIY interneurons (H. Murakami et al., 2005). Since the distal part of the neurites of AIY, which mainly contain postsynapses (White et al., 1986), are truncated in *daf-16* mutants (Christensen, de la Torre-Ubieta, Bonni, & Colon-Ramos, 2011), defective transmission from AFD sensory neurons to AIY interneurons might be manifested in behavior in the *Lb. reuteri*-fed D5 condition.

Bacterial screen to address age-dependent phenotypes

Even with *C. elegans* with a short lifespan, it is challenging to address age-dependent neuronal phenotypes because powerful forward genetic screens are not readily applicable to aged worms. Our study showed that bacterial screen is useful for generating phenotypic diversity and address underlying

molecular mechanisms in aged animals. The bacterial screen has been applied to various *C. elegans* phenotypes. Watson *et al.* carried out unbiased mutant screens of *Escherichia coli* and *Comamonas aquatica* to identify bacterial genes that affect the "dietary sensor" in *C. elegans*, which increases the GFP intensity when fed *Comamonas*; they found that mutations in genes involved in vitamin B12 biosynthesis/import increased *C. elegans* dietary sensor activity (Watson et al., 2014). Zhou et al. screened 13 LAB and found that *Lactobacillus zeae* protects *C. elegans* from enterotoxigenic *E. coli* (M. Zhou et al., 2014). Given that *C. elegans* has its natural microbiota (Berg et al., 2016; Dirksen et al., 2016; Samuel et al., 2016; Zhang et al., 2017), the nervous system of worms in a natural environment may be affected by complex bacteria. Indeed, a recent study has revealed that tyramine produced from commensal bacteria affect *C. elegans* avoidance behavior (O'Donnell et al., 2020). Hence, bacterial screens will provide a unique angle of understanding for *C. elegans* research.

Materials and Methods

Worm maintenance and strains

C. elegans strains were maintained at 23°C on Nematode Growth Medium (NGM) plates with *E. coli*, OP50, as previously reported (Brenner, 1974). N2 (Bristol) was used as the wild type. The following mutant strains were used for thermotaxis assays: DA1116 *eat-2(ad1116)*; CF1038 *daf-16(mu86)*; IK0656 *tax-6(db60)*; NUJ69 *kmo-1(tm4529)*; NUJ71 *nkat-1(ok566)*. NUJ69 *kmo-1(tm4529)* is a one-time outcrossed FX04529 *kmo-1(tm4529)* strain. NUJ71 *nkat-1(ok566)* is a two-time outcrossed RB784 *nkat-1(ok566)* strain.

Preparation of bacterial plates

E. coli, OP50, was inoculated into Luria-Bertani (LB) broth, cultured overnight at 37 °C. LAB strains were provided by Megmilk Snow Brand company (Table S1). Bacteria were inoculated into the liquid medium from glycerol stocks and cultured in the conditions described in Supplementary Table 1. Bacterial cells were collected by centrifugation at 7,000x g for 10 min at 4 °C. Cells were washed twice with sterile 0.9% NaCl solution. The washed bacteria were adjusted to a final concentration of 0.1 g/ml (wet weight) in NG buffer (25 mM K-PO_4 (pH6), 50mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$). For heat killing, 0.1 g/ml bacteria in tubes were incubated for 1 h in a 65 °C incubator for *E. coli* or in boiling water for LAB. By this treatment, bacterial colony-forming unit (cfu) became $<1.0x10^2$ cfu/ml, which is at least 10^8 lower than live bacteria ($>1.5x10^{10}$). Two hundred microliters of the bacterial suspension were spread onto 60-mm NGM plates and dried overnight. NGM plates with peptone were used except for lifespan assays, where NGM plates without peptone were used.

Preparation of aged worms fed different bacteria

For behavioral assays, synchronized eggs were prepared by bleaching gravid hermaphrodites using 0.5x household bleach in 0.5 M NaOH and placed onto NGM plates with OP50. The eggs were cultured at

23 °C for 72 hours to obtain day one adults (D1) unless otherwise noted. For thermotaxis of aged worms, day one worms were washed with M9 and transferred to NGM plates with OP50 or LAB every day. For thrashing assay and locomotion assay, worms were transferred individually by picking.

Thermotaxis assay

Population thermotaxis assays were performed as described (Ito et al., 2006). Fifty to 250 worms on cultured plates were washed with M9 and placed at the center of the assay plates without food and with a temperature gradient of 17-23 or 20-26 °C. The temperature gradient was measured to be ~0.5 °C/cm. After letting them move for 1 h, the number of adult worms in each of eight sections along the temperature gradient (Fig. 1A) was scored under a stereomicroscope. The fraction of worms in each section was plotted on histograms. The performance index and thermotaxis index were calculated, as shown in Fig. 1A.

Thrashing assay

Thrashing assay was performed, as previously described with a few modifications (Tsalik & Hobert, 2003). Worms were washed with NG buffer and transferred with a drop of NG buffer onto an NGM plate without food using a capillary pipet. In liquid, worms show lateral swimming movements (thrashes). We defined a single thrash as a complete movement through the midpoint and back and counted the number of thrashes for 30 seconds.

Motility assay

Assay plates were prepared by placing circular filter paper with a one-inch hole on NGM plates with OP50 or LAB and soaking the paper with ~100 µl of 100 mM $CuCl_2$. A single worm was transferred to an assay plate with the cultured bacteria and left at 23 °C for three minutes. The images of the bacterial lawn were captured by a digital camera (Fujifilm) through an eyepiece of a stereomicroscope, Stemi 508 (Zeiss). The trajectory of a worm on the lawn was traced using FIJI (Schindelin et al., 2012) and

395 measured as the distance of locomotion. The distance (mm) was divided by time (min) to calculate the
396 speed.
397
398 **Food recognition assay**
399 Food recognition assay was performed as previously described with a few modifications (Sawin et al.,
400 2000). Assay plates were prepared by spreading OP50, as described for worm maintenance. For well-fed
401 animals, worms were washed twice in S basal buffer (Brenner, 1974), and transferring them to an assay
402 plate in a drop of the buffer using a capillary pipette. Five minutes after transfer, the number of body
403 bends in 20 s intervals was counted. For starved animals, 5–15 animals were washed twice in S basal
404 buffer and incubated on NGM plates without food for 30 min. The number of body bends was measured
405 as described above for well-fed animals.
406
407 **Lifespan assay**
408 Worms were synchronized by bleaching gravid adults and grown with regular NGM plates with OP50
409 until day 1 of adulthood. Day1 adults were washed three times with M9 buffer and transferred to peptone-
410 free NGM plates supplemented with 50 mg/ml OP50 or LAB. Worms were transferred to new plates
411 every day until they became D4 adults, and every other day afterward. Dead worms were defined as no
412 voluntary movement after several touches on the head and tail and counted every day. Four independent
413 sessions with 25 worms per session were combined for each condition.
414
415 **Quantitative RT-PCR**
416 Non-gravid young adult worms were used as D1 to avoid contamination of eggs. D5 worms fed *E. coli* or
417 LAB were prepared as described above. Total RNA was extracted from whole worms using RNAiso Plus
418 reagent (Takara). Two micrograms of total RNA were reverse transcribed into cDNA with a mixture of
419 random and oligo dT primers using ReverTra Ace qPCR RT Master Mix with gDNA Remover
420 (TOYOBO). The cDNA and gene-specific primers were used for qPCR reaction with THUNDERBIRD

SYBR qPCR Mix (TOYOBO), and the products were detected using a LightCycler 96 System (Roche). The following primers were used: *pha-4* (KN1370: 5'-GGTTGCCAGGTCCCCTGACA-3' and KN1371: 5'-GCCTACGGAGGTAGCATCCA-3'); cdc-42 is used as a reference because it is stable and unaltered during aging (Hoogewijs, Houthoofd, Matthijssens, Vandesompele, & Vanfleteren, 2008; Mann, Van Nostrand, Friedland, Liu, & Kim, 2016) (KN1170: 5'-CTGCTGGACAGGAAGATTACG-3' and KN1171: 5'-CTCGGACATTCTCGAATGAAG-3').

Phylogenetic tree

16S rRNA sequences of LAB were obtained from the Genome database of NCBI (http://www.ncbi.nlm.nih.gov/genome/), and the accession numbers are shown in Table S2. The phylogenetic tree was inferred by the Neighbor-Joining method based on the 16S rRNA gene sequence of model LAB strains. The evolutionary distances were computed using the Maximum Composite Likelihood method conducted in MEGA X.

Gram staining

Bacteria are fixed with methanol and stained using Gram Color Kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Stained bacteria are imaged using an Axio Imager.A2 equipped with a Plan-Apochromat 63x/1.4 oil objective (Zeiss).

Statistical analyses

Box-and-whisker plots represent medians as center lines; boxes as first and third quartiles; whiskers as maximum and minimum values except for outliers, which are 1.5 times greater than the upper limit or 1.5 times smaller than the lower limit of the interquartile range; dots as outliers. We used Student's t-test to compare two samples and one-way or two-way ANOVA followed by Dunnett's test to compare multiple samples using R (R core team, https://www.R-project.org/, Vienna, Austria) or GraphPad Prism 7.0

(GraphPad Software, La Jolla, CA). In all figures, *p < 0.05, **p < 0.01, and p >0.05 is considered as not significant (ns).

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Competing interests

S.H. is an employee of Bean Stalk Snow Co., Ltd., and S.T. is an employee of Megmilk Snow Brand Co., Ltd. The other authors declare no competing interests.

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676

677 **Figure legends**

678 **Figure 1 Thermotaxis performance declines with age**

679 (A) Schematic of thermotaxis assay. Worms are placed at light blue circles on a thermal gradient without
680 food. The pink rectangle indicates the sections around the T_{cult}. The number of worms in each section
681 after one hour was used to calculate the performance index using the indicated formula. (B and C) Age-
682 dependent changes in thermotaxis behavior. D1 and D5 worms were cultivated at 20 °C and placed at the
683 center of 14-20 or 20-26 °C gradient. (B) and (C) indicate distributions of worms (pink rectangle: the
684 sections around the T_{cult}) and box plots of performance indices, respectively. Numbers of experiments are
685 shown. Statistics: Student's t-test compared to D1 adults. **p<0.01, ***p<0.001; ns, p>0.05. (D) Box
686 plots of performance indices of worms at different ages. Numbers of experiments are shown. Statistics:
687 One-way ANOVA followed by Dunnett's multiple comparison test compared to D1 adults. **p<0.01,
688 ***p<0.001; ns, p>0.05.

689

690 **Figure 2 LAB screen for thermotaxis in aged worms**

691 (A) Schematic of the screening procedure. Worms were cultivated at 23 °C with *E. coli* until D1 and
692 transferred to new *E. coli* or LAB plates every day. At D5, worms were subjected to thermotaxis assay
693 with a thermal gradient of 17-23 °C. (B) Box plots comparing performance indices of D5 worms fed LAB
694 to those of D1 (pink dashed line) and D5 worms (light blue dashed line) fed *E. coli*. Not applicable (NA)
695 indicates that worms fed those LAB were not subjected to the assay because they were sick or dead.
696 Abbreviations: B, *Bifidobacterium*; Lb, *Lactobacillus*; Lc: *Lactococcus*; Ls, *Leuconostoc*; P,
697 *Pediococcus*; S, *Streptococcus*. Numbers of experiments are shown. Statistics: One-way ANOVA
698 followed by Dunnett's multiple comparison test compared to D5 adults fed *E. coli*, ***p<0.001;
699 **p<0.01; *p<0.05.

700

701 **Figure 3 Aged worms fed LAB remembers new T_{cult}**

(A and B) The distribution of worms fed indicated bacteria are shown. Pink rectangles indicate the sections around the T_{cult}. (A) Worms were cultivated at 23 °C and placed at the center of the 17-23 °C gradient. (B) Temperature shift assay. T_{cult} was shifted from 23 °C to 17 °C one day before the assay. Worms were placed at the center of the 17-23 °C gradient. (C) Box plots summarizing thermotaxis indices corresponding to (A) and (B). Numbers of experiments are shown. Statistics: One-way ANOVA followed by Dunnett's multiple comparison test compared to D5 adults fed *E. coli*, ***p<0.001; **p<0.01; *p<0.05; ns, p>0.05. Student's t-test for comparison between T_{cult}=23 °C and T_{cult}=23 °C → 17 °C, ###p<0.001; ##p<0.01; #p<0.05; ns, p>0.05.

Figure 4 Aged worms sense food normally

(A) Box plots show performance indices of worms fed indicated bacteria and cultivated at 23 °C. Aged worms were transferred every day to new plates from D1. Numbers of experiments are shown. Statistics: One-way ANOVA followed by Dunnett's multiple comparison test compared to D5 adults fed *E. coli*, ***p<0.001; ns, p>0.05. (B and C) Food recognition assays of D1 in (B) and D5 adults in (C). Worms were pre-conditioned with or without *E. coli* and assayed on plates with or without *E. coli*. Worms locomotion was evaluated by body bends in 20 sec. The presence of food on the assay plate slows down the locomotion of well-fed worms (basal slowing response). Pre-conditioning worms without food enhanced the basal slowing response (enhanced slowing response). The numbers of worms examined are shown. Error bars: S.E.M. Statistics: Two-way ANOVA with Turkey's multiple comparison test, ***p<0.001; **p<0.01; ns, p>0.05.

Figure 5 LAB show various effects on locomotion and lifespan

Worms were fed indicated bacteria from D1. (A) Survival curves of worms fed indicated LAB are shown with control worms fed *E. coli*. NGM plates without peptone were used to avoid the undesired growth of *E. coli* on LAB plates. n=4 experiments with 25 worms/experiment. Statistics: Log-rank test. p values are shown. (B and C) The number of thrashes in liquid (B) and distance of migration in three minutes on

plates with food (C) were measured to quantitate the locomotion of aged worms. The numbers of worms are shown in bars. Error bars: S.E.M. Statistics: One-way ANOVA followed by Dunnett's multiple comparison test compared to D5 fed *E. coli*, p***<0.001; ns, p>0.05.

Figure 6 *Lactobacilli* in a clade are associated with high thermotaxis performance of aged worms

(A) Phylogenetic tree of LAB based on 16S rRNA is shown with fermentation mode and heatmap of performance indices of aged worms fed indicated LAB from D1. Bootstrap values are indicated at each node on the phylogenetic tree. Fermentation modes were categorized based on previous studies (see Table S1). For performance indices, the same data as Figure 2A were used. NA in the performance indices heatmap indicates that worms fed those LAB were not subjected to the assay because they were sick or dead. Fermentation mode indicates obligatory hetero- (green), facultatively hetero- (light green), and obligatory homofermentative (orange) LAB. (B) Performance indices are shown for clades of LAB. Each dot indicates one LAB species. *Lactobacillus* is separated into two clades, A and B as shown in (A). Statistics: The mean indices marked with distinct alphabets are significantly different ($p < 0.001$) according to One-way ANOVA followed by Tukey–Kramer test. (C) Box plots summarizing performance indices of aged worms fed either live or dead bacteria. Numbers of experiments are shown. Statistics: The mean indices marked with distinct alphabets are significantly different ($p < 0.05$) according to One-way ANOVA followed by Tukey–Kramer test.

Figure 7 *daf-16* is involved in the effect of *Lb. reuteri* on thermotaxis in aged worms

(A) Expression of *pha-4* transcripts in aged worms fed indicated LAB relative to aged worms fed *E. coli*. (B) Box plots summarizing thermotaxis indices of wild type and *eat-2* mutant worms cultivated at 23 °C with *E. coli*. Numbers of experiments are shown. Statistics: Student's t-test, ns, p>0.05. (C-E) Worms with indicated genotypes were cultivated at 23 °C with *E. coli* or *Lb. reuteri*. (C) Box plots summarizing performance indices. Numbers of experiments are shown. Statistics: One-way ANOVA followed by Dunnett's multiple comparison test compared to the D1 control for each condition, p***<0.001; ns,

754 p>0.05. (D) Distribution of worms. Pink rectangles indicate the sections around the T_{cult}. (E) Survival

755 curves. NGM plates without peptone were used. n=4 experiments with 25 worms/experiment. Statistics:

756 Log-rank test, p***<0.001; ns, p>0.05.

Supplementary information

Supplementary Figure Legends

Figure S1 Survival curve of worms cultivated at different temperature

Survival curves of worms cultivated at the indicated temperature from eggs. Worms were fed *E. coli* on NGM plates. n=4 experiments with 25 worms/experiment. Statistics: Log-rank test, p***<0.001.

Figure S2 Distributions of worms on the temperature gradient at different age

Worms were cultivated at 23 °C and placed at the center of the 17-23 °C gradient. The distributions of worms were shown. Pink rectangles indicate the sections around the T_{cult}.

Figure S3 Worms fed select LAB were not thermophilic

Worms were cultivated at 23 °C and placed at the center of 20-26 °C gradient. (A) Distribution of worms fed indicated bacteria. Pink rectangles indicate the sections around the T_{cult}. (B) Box plots summarizing thermotaxis indices of aged worms fed indicated conditions. Numbers of experiments are shown. Statistics: The mean indices marked with distinct alphabets are significantly different ($p < 0.05$) according to One-way ANOVA followed by Tukey–Kramer test.

Figure S4 Peptone-free plates do not affect the beneficial effect of *Lb. reuteri*

Box plots comparing performance indices between worms cultivated on NGM plates without peptone. Worms were cultivated at 23 °C with *E. coli* or *Lb. reuteri*. Numbers of experiments are shown. Statistics: One-way ANOVA followed by Dunnett's multiple comparison test compared to the D1 control for each condition, p***<0.001; ns, p>0.05.

Figure S5 Images of Gram-stained bacteria

781 Representative images of Gram-stained *E. coli* and select LAB. *E. coli* and LAB are Gram-negative and

782 positive, respectively. Scale bar: 10 μm.

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784 **Table S1 List of LAB strains**

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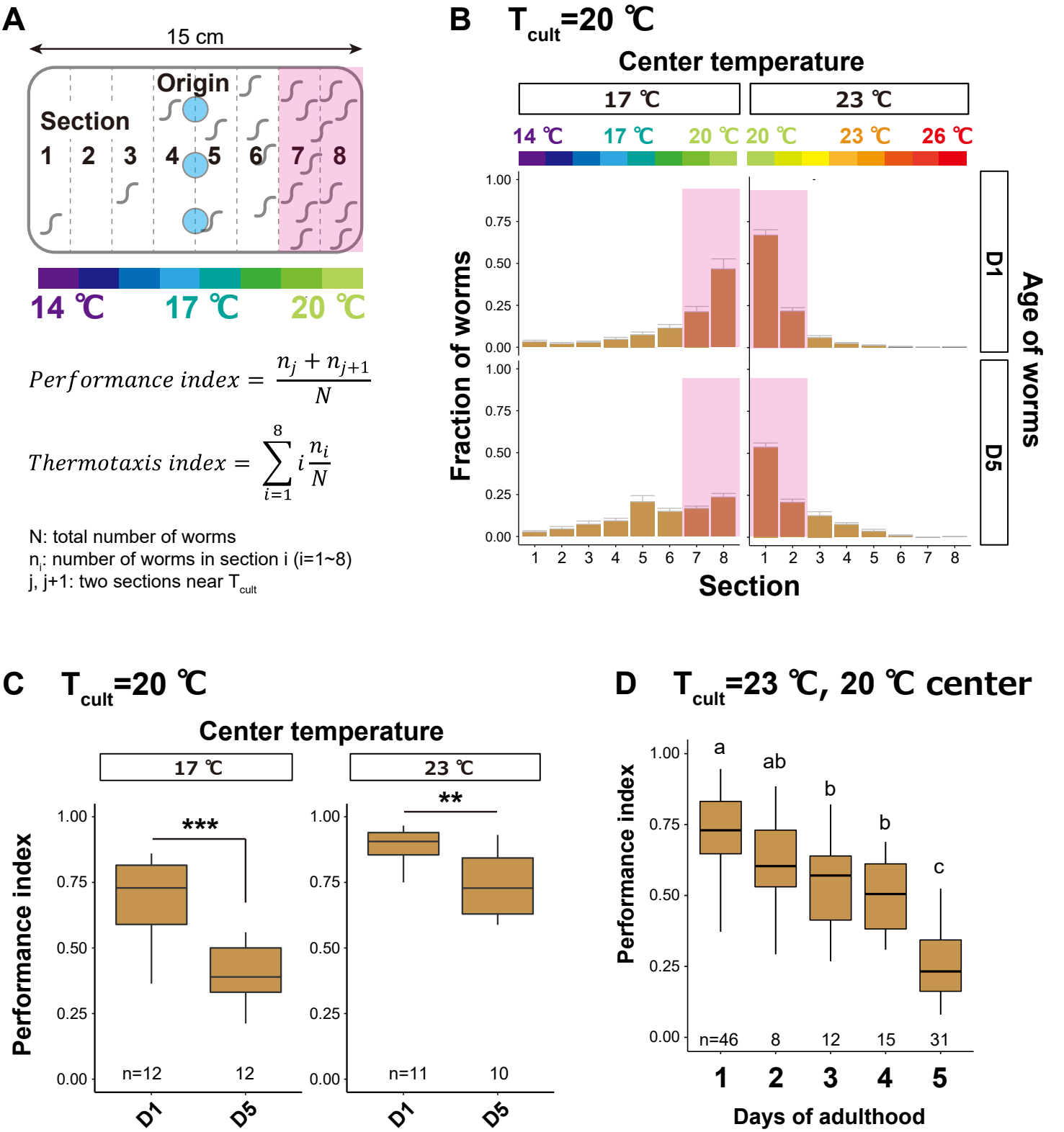

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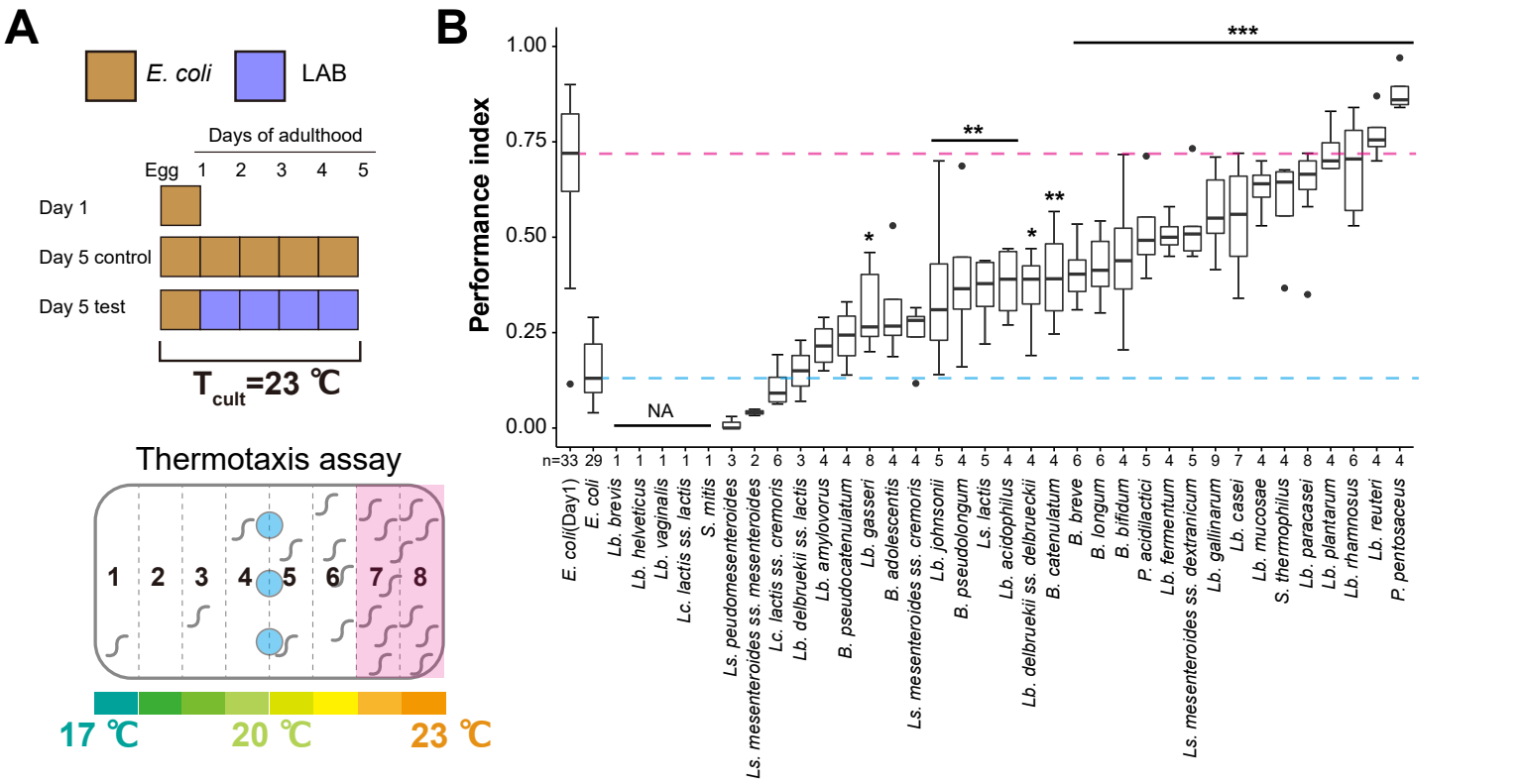

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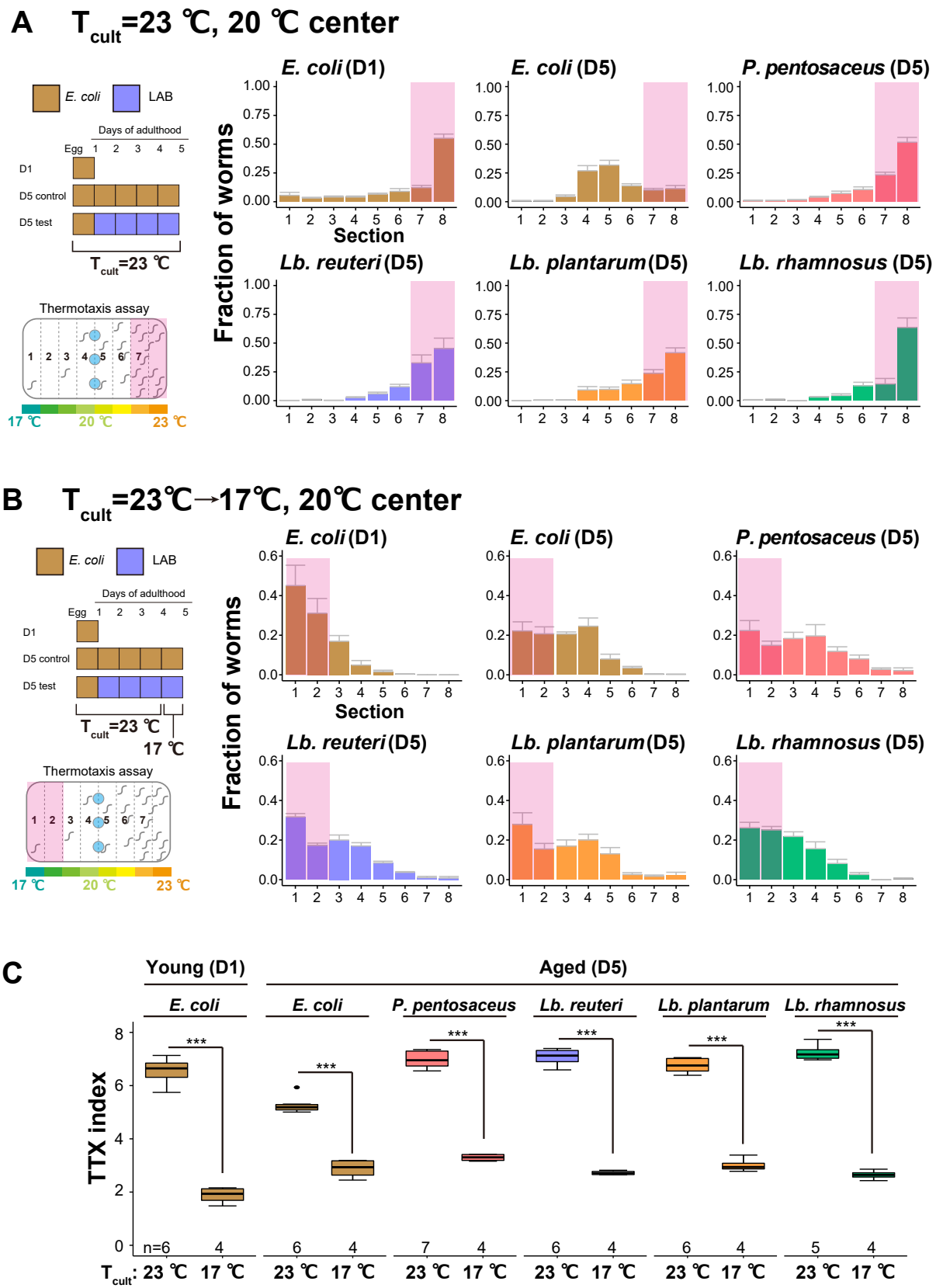

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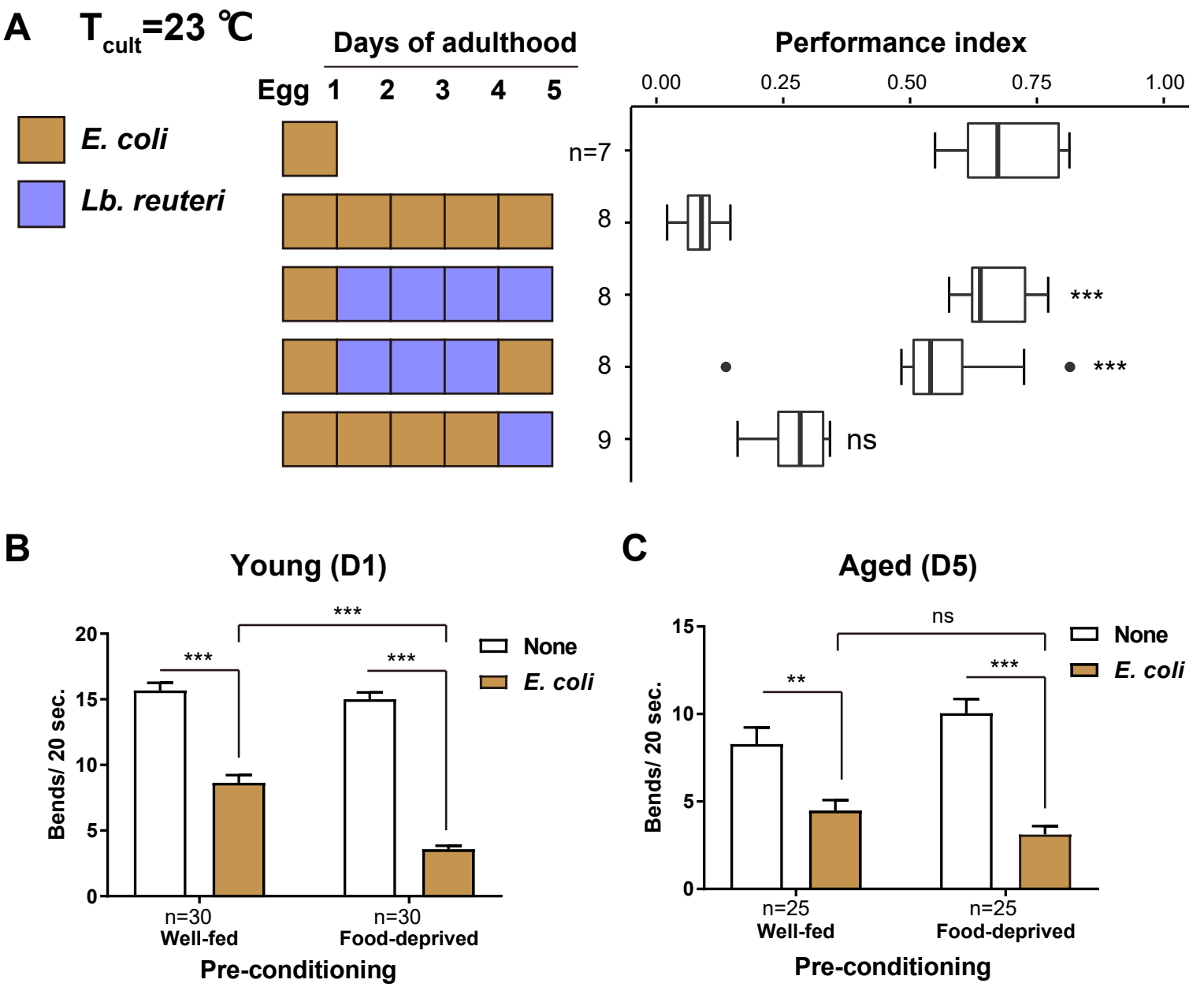

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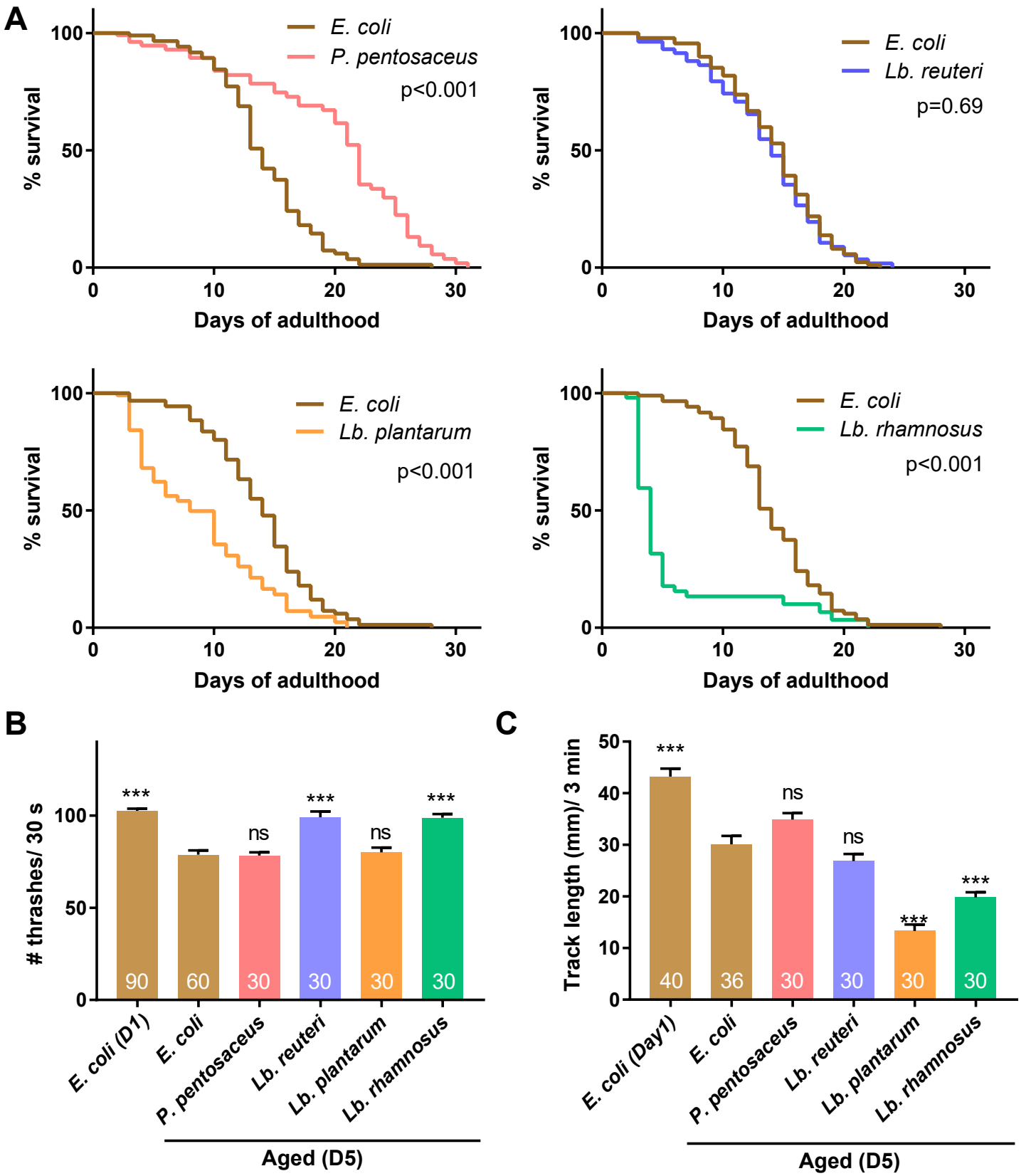

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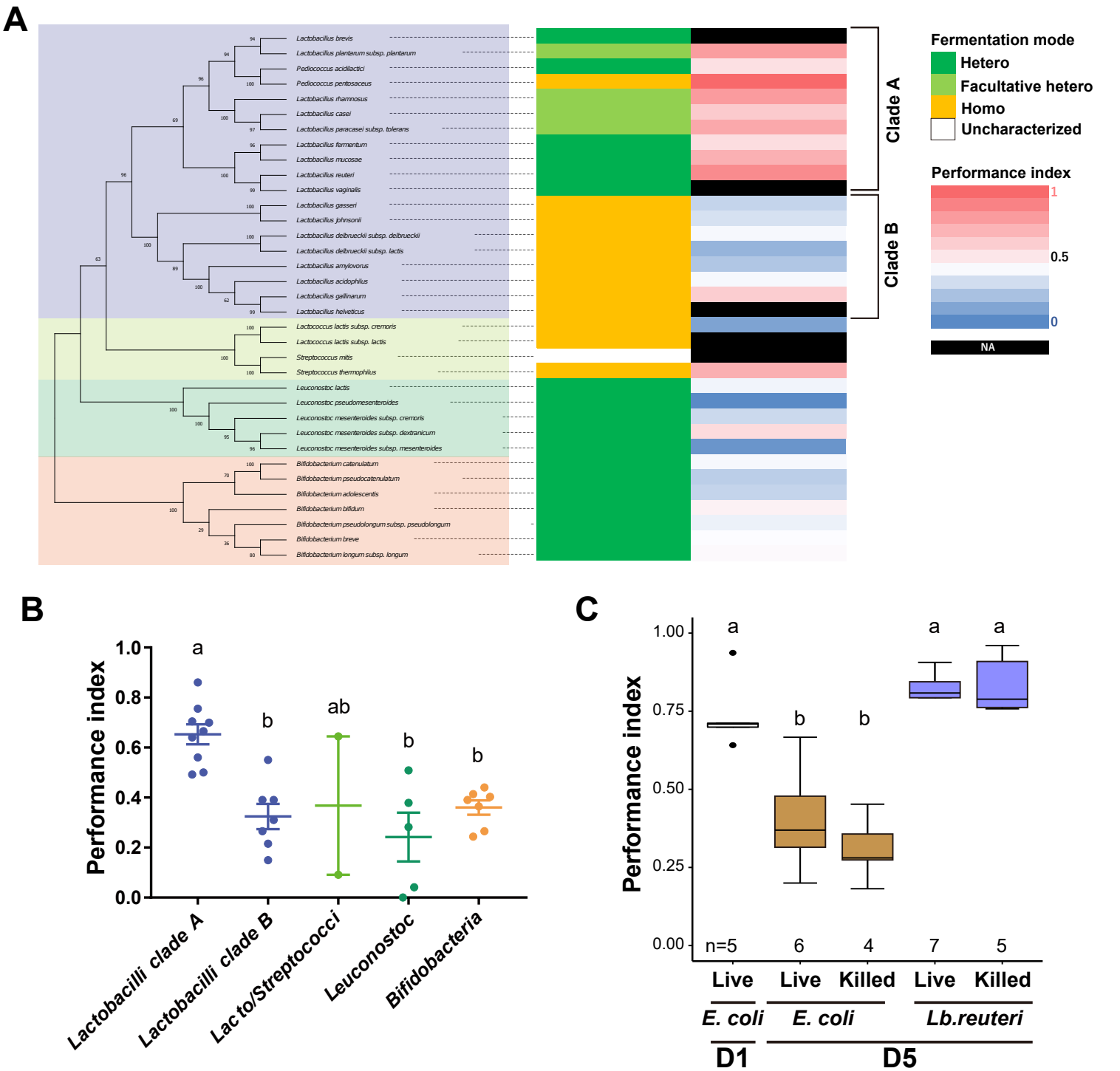

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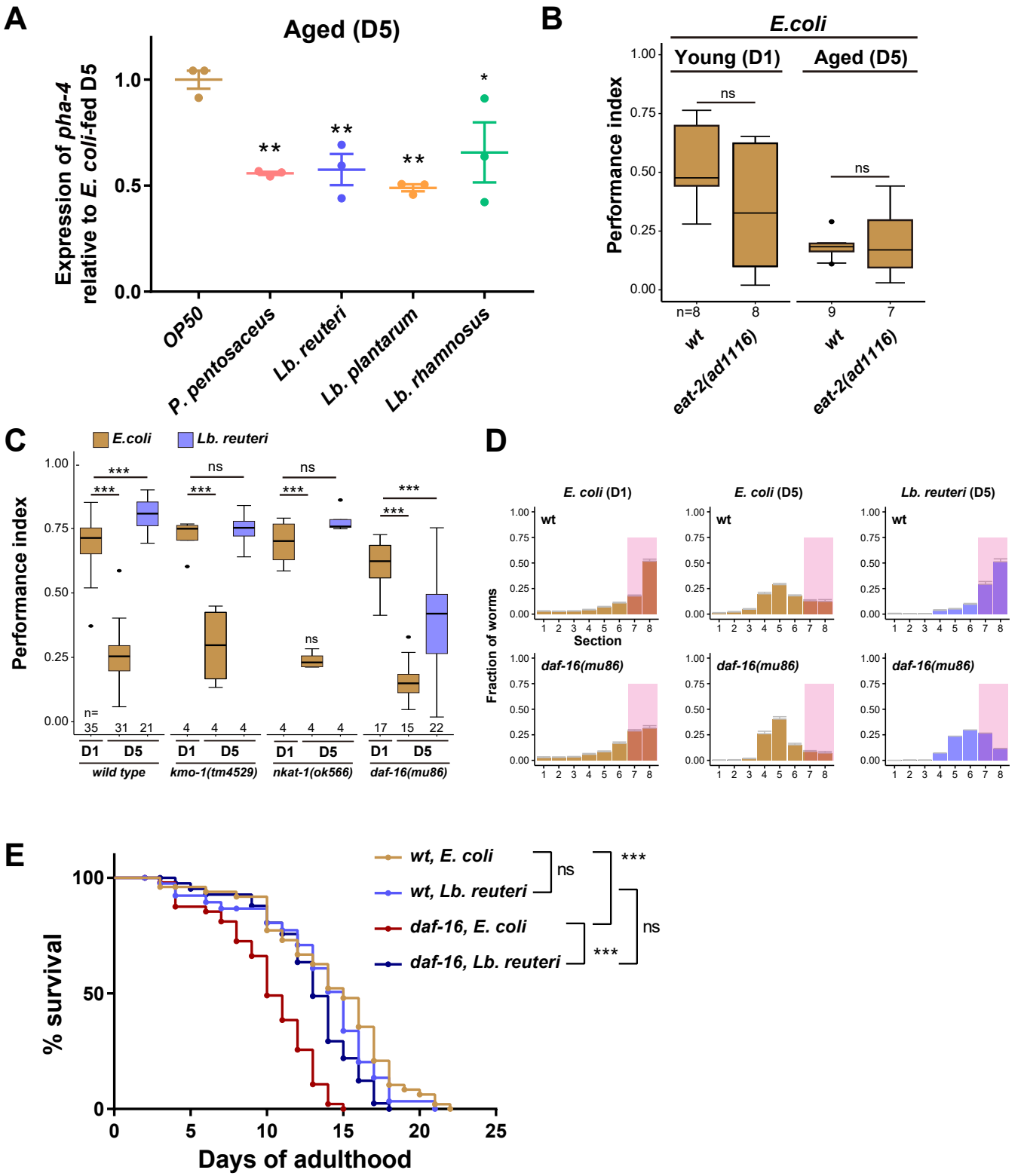

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