APPLIED MICROBIAL AND CELL PHYSIOLOGY

Effects of *Lactobacillus salivarius*, *Lactobacillus reuteri*, and *Pediococcus acidilactici* on the nematode *Caenorhabditis elegans* include possible antitumor activity

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Abstract This study examined the effects of three lactic acid bacteria (LAB) strains on the nematode *Caenorhabditis elegans*. *Lactobacillus salivarius*, *Lactobacillus reuteri*, and *Pediococcus acidilactici* were found to inhibit the development and growth of the worm. Compared to *Escherichia coli* used as the control, *L. reuteri* and *P. acidilactici* reduced the lifespan of wild-type and short-lived *daf-16* worms. On the contrary, *L. salivarius* extended the lifespan of *daf-16* worms when used live, but reduced it as UV-killed bacteria. The three LAB induced the expression of genes involved in pathogen response and inhibited the growth of tumor-like germ cells, without affecting DAF16 localization or increasing corpse cells. Our results suggest the possible use of *C. elegans* as a model for studying the antitumor attributes of LAB. The negative effects of these LAB strains on the nematode also indicate their potential use against parasitic nematodes.

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Introduction

Many strains of lactic acid bacteria (LAB) have been described and exploited commercially as probiotics (Gerritsen et al. 2011). The positive effects of these strains on animal and human health, that include improvement of digestion and nutrient uptake, increased resistance to pathogenic bacteria, antitumor effects, and increased longevity, have been the subject of many reviews (Gerritsen et al. 2011; Taverniti and Guglielmetti 2011; Ottaviani et al. 2011).

The mechanisms behind the probiotic effects of bacteria are not entirely understood. Probiotic bacteria have been shown to compete against other microorganisms that are found within the intestine and may be pathogenic. This is most likely achieved through the secretion of bacteriocins (Gerritsen et al. 2011). There is now evidence to suggest that the beneficial modulation of the intestinal microbiota could also protect against colorectal cancer (Candela et al. 2011). Production of hydrogen peroxide by LAB has been shown to be effective against food-borne pathogens (Nakajima et al. 2003). LAB have also been shown to stimulate local epithelial innate immune responses that prevent intestinal inflammation in a Crohn's disease mouse model, as well as production of TNF-α and activation of NF-κB in vitro (Pagnini et al. 2010). In BALB/c mice fed LAB, a dose-dependent increase of the Bcl2 protein was observed, and an analysis of cytokine-producing cells in the lamina propria of the gut showed increased TNF-α and INF-γ values (Perdigón et al. 2002). Strain-specific transcriptional responses to LAB, involving the Toll-like receptor pathway,

have also been reported using human intestinal epithelial cell cultures (Putaala et al. 2010).

Caenorhabditis elegans is susceptible to a wide range of pathogens and has been proven to be a very useful model for studying host–microbe interactions and virulence, providing many genetic and molecular tools for this purpose (Aballay et al. 2000; Labrousse et al. 2000; Garsin et al. 2001; Couillault and Ewbank 2002; Zhang et al. 2005; Tan and Shapira 2011). Virulence mechanisms have been shown to be highly conserved among divergent hosts (Mahajan-Miklos et al. 2000) and the worm has been used to study a wide range of pathogens such as *Salmonella enterica*, *Pseudomonas aeruginosa* (Alegado et al. 2003), and *Leigonella pneumophila* (Komura et al. 2010).

It is reasonable to consider that *C. elegans* should be useful in providing insights regarding the mechanisms by which probiotic bacteria exert beneficial effects. Little work has been done in this area, and so far, it has been shown that *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* were able to extend the worm's lifespan as well as increase resistance to *Salmonella enterica* (Ikeda et al. 2007). Similarly, Komura et al. (2010) demonstrated the protective effects of *B. infantis* against *L. pneumophila*. Moreover, Wang et al. (2011) have suggested the use of *C. elegans* for preselecting *Lactobacillus* isolates to control *Salmonella* infections.

The aim of this study was to examine the effect of LAB on growth, aging, immune response, and tumor-like growth in *C. elegans*. For this purpose, the following three LAB strains were used: *Lactobacillus reuteri* (LR), a common LAB strain found in the intestine of humans that produces reuterin, a broad spectrum microbial inhibitor (Talarico et al. 1988; Casas and Dobrogosz 2000); *Lactobacillus salivarius* (LS) that has been shown to have immunomodulatory effects in mice and can also stimulate the maturation of immature mouse spleen dendritic cells and inhibit the in vitro infectivity of HIV-1 (Galvez et al. 2008; Langa et al. 2012); and *Pediococcus acidilactici* (PA) that has been shown to modulate intestinal bacterial communities in ongrowing red tilapia and stimulate some aspects of the non-specific immune response (Ferguson et al. 2010) as well as suppress autoimmune encephalomyelitis by inducing IL-10-producing regulatory T cells (Takata et al. 2011). LAB were compared to the standard food source of *C. elegans*, *Escherichia coli* strain OP50 (OP).

Materials and methods

Worm and bacterial strains used

L. salivarius DSM 20555, *L. reuteri* DSM 20016, and *P. acidilactici* DSM 20284 were obtained as lyophilized stock cultures (DSMZ GmbH, Braunschweig, Germany). *C. elegans* strains used were N2 (wild-type), CF1038 *daf-16 (mu86)*, JK1466 *gld-1(q485)/dpy-5(e61);unc-13(e51)*, MT2547 *ced-4(n1162)*, and CF1407 *daf-16(mu86); muIs71*[pKL99(*daf-16a*::GFP/bKO) + pRF4(rol-6)] purchased from the Caenorhabditis Genetics Center (CGC, St Paul, MN, USA). *E. coli* OP50 was used as the standard food source for growing and maintaining *C. elegans*.

LAB cultures and worm plates

LS and LR were grown on MRS (Sigma-Aldrich, St. Louis, MO, USA) agar plates supplemented with Tween 20 at 37 °C; PA was grown on MRS agar plates supplemented with Tween 20 at 30 °C; and OP was grown on LB plates at 37 °C. Because the medium used to grow bacteria can influence their effect on *C. elegans* (Garsin et al. 2001), all cultures used to seed nematode growth medium (NGM) plates (Stiernagle 2006) were grown overnight in MRS broth supplemented with Tween 20, at 30 or 37 °C accordingly. NGM agar plates were then seeded with 0.25–0.5 ml of bacterial culture and incubated overnight at 30 or 37 °C. For experiments using killed bacteria, seeded plates were UV-irradiated.

Longevity assays

Worms grown at 20 °C on NGM plates seeded with OP were hypochloride-treated (Stiernagle 2006) to remove any live bacteria and moved to plates with UV-killed OP. Worms from these plates were used to create synchronized cultures (Sutphin and Kaeberlein 2009). One-day-old adult worms (30 per plate) were transferred to NGM plates seeded with live or UV-killed cultures of OP, LS, LR, or PA. The plates were kept at 20 °C and the worms were transferred every 2 days to fresh plates. During transfer, worms were scored as dead if they failed to respond when touched with a pick. Worms that crawled off the agar or died as a result of internal hatching were removed from the experiment.

Body size

N2 worms grown on plates seeded with UV-killed OP were moved to live OP or LAB plates as 1-day-old adults. After 3, 7, and 11 days, the worms were immobilized in M9 buffer (Stiernagle 2006) containing 25 mM levamisole on agar pads and photographed using a Leica M205 FA stereoscope. The body sizes were calculated using ImageJ software (Abramoff et al. 2004). Each data point represents the mean size of at least ten worms with standard errors.

Intestinal colonization

The measurement of bacteria within the worm's intestinal tract was performed according to Garsin et al. (2001). Briefly, N2 worms grown on plates seeded with UV-killed OP were moved to live OP or LAB plates as 1-day-old adults. After 2 days, worms were collected and washed four times in M9 buffer. Three worms (per sample) were placed in a microcentrifuge tube containing 20 μl M9 containing 1 % Triton X-100 and mechanically disrupted using a pestle. A series of dilutions were plated on LB agar (OP) or MRS agar (LAB). Each data point represents the mean cfu from triplicate samples with standard errors.

Confocal microscopy

Synchronized 1-day-old adult N2 and *ced-4* and *gld-1* worms were moved from UV-killed OP plates to live OP and LAB plates. After 1–5 days, nuclei visualization was achieved by staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Briefly, worms were washed in M9 and fixed in methanol for 20 min at −20 °C. Samples were washed again in M9, incubated for 15 min in 1 mg ml^{-1} DAPI in M9 at RT, washed in M9, and mounted on 3 % agarose pads for microscopy. For visualizing corpse cells, the worms were stained with SYTO 12 (Molecular Probes, Eugene, OR, USA) as previously described (Gumienny et al. 1999). Briefly, worms were washed in M9 and incubated in 33 μM SYTO 12 in M9 for 4–5 h at RT. Worms were then washed in M9 and transferred to fresh OP plates. After 30 min, worms were anesthetized in levamisole and mounted on 3 % agarose pads for microscopy.

Synchronized 1-day-old adult transgenic worms expressing DAF16::GFP were moved from UV-killed OP plates to live OP and LAB plates. After 24 h, worms were anesthetized in levamisole and mounted on 3 % agarose pads. All samples were visualized using a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Transmission electron microscopy

Wild-type N2 worms were grown to L4 stage on UV-killed OP and then moved to live OP and LAB plates. Five days later, samples for transmission electron microscopy (TEM) were prepared by fixing the worms in 0.1 M KPO_4 buffer containing 2.5 % glutaraldehyde, 1 % paraformaldehyde, and 0.1 M sucrose for 2 h at 4 °C. Samples were washed in 0.2 M KPO_4 buffer and incubated for 90 min at 4 °C in 0.5 % OsO_4 in 0.1 M KPO_4 buffer. Samples were washed again in 0.2 M KPO_4 buffer and enclosed in 3 % agarose cubes. Dehydration and UAc staining took place by incubating for 10 min in 30 and 50 % acetone, 30 min in 70 %

acetone containing 1 % UAc, and 10 min in 95 and 100 % acetone. Samples were then placed in freshly made resin and cured for 3 days. Sections were cut using a Reichert OMU3 ultramicrotome and visualized using a JEOL JEM-100S TEM (JEOL Ltd, Tokyo, Japan).

Quantitative real-time PCR

One-day-old adult N2 worms were moved from UV-killed OP plates to live OP and LAB plates. After 24 h at 20 °C, worms were washed in M9 and total mRNA was isolated using RNAzol (Sigma-Aldrich), according to the manufacturer's instructions. RNA was transcribed to cDNA using iSctipt cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) with random primers. Quantitative real-time PCR was performed using a Bio-Rad MJ Mini PCR unit equipped with a Mini Opticon Detection System using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories). Calculation of reaction efficiencies and analysis of expression data were performed using CFX manager™ software (Bio-Rad Laboratories). Primers were designed using Primer3 (http://primer3.sourceforge.net/). Primer sequences are available upon request.

Statistical analysis

Calculation and comparison of mean lifespans was done using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and the log-rank (Mantel–Cox) test. Body size and gene expression results were compared by one-way analysis of variance using GraphPad Prism 5.00.

Results

Effect on fertility and growth

When adult worms were moved to plates seeded with LAB, the first eggs they laid hatched and arrested as L1s. Eggs laid after a few hours did not hatch at all. In fact, all larval stages arrested when they were introduced to LAB plates except for L4s that developed into adults but with a significantly ($P \leq 0.05$) smaller body size compared to those fed OP (Fig. 1).

Intestinal colonization and TEM

In order to verify that LAB were consumed by the worms, intestinal colonization experiments were performed and showed that LAB cells were indeed present within the intestinal track (Fig. 2). Bacterial cells being digested were also visible in images of the intestine produced by TEM
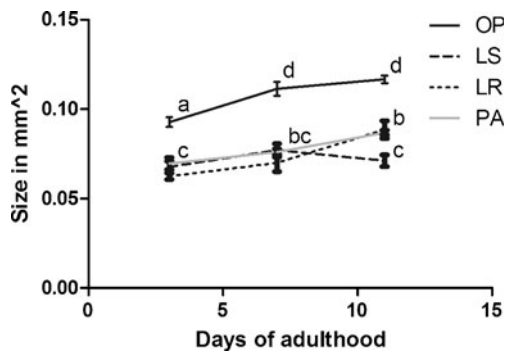
Fig. 1 Body size of worms moved to LAB plates as L4 larvae and measured after 3, 7, and 11 days. N2 worms grown on plates seeded with UV-killed OP were moved to live OP or LAB plates as 1-day-old adults. Worms photographed and the body size was calculated using ImageJ. Each data point represents the mean size of at least ten worms ± standard errors. Points next to different superscripts (*a–d*) differ significantly (*P*<0.05)

(Fig. 3). TEM images of the body wall muscle of 6-day-old adult worms fed OP or LAB did not reveal any morphological differences between treatments (data not shown).

Effect on longevity

As the nematodes did not develop on the LAB plates, the longevity experiments began using 1-day-old adults. The results showed that the LAB significantly (*P*<0.01) reduced the lifespan of WT (N2) worms (Fig. 4a). UV-killed LS and LR further reduced the worm's lifespan while UV-killed PA did not significantly (*P*>0.05) affect it (Fig. 4b).

For LR and PA, the use of short-lived *daf-16* mutants revealed an even greater sensitivity to the LAB indicating that the insulin/insulin-like signaling cascade may play a role in this effect (Fig. 5a). However, LS significantly
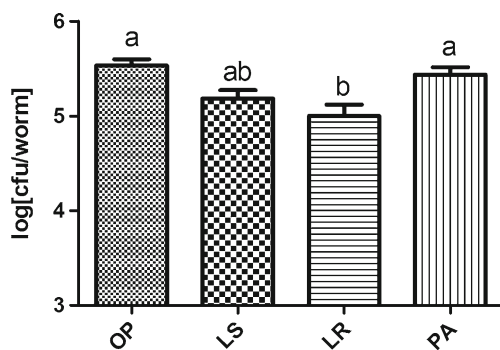

Fig. 2 Numbers of live bacterial cells recovered from worms. N2 worms grown on plates seeded with UV-killed OP were moved to live OP or LAB plates as 1-day-old adults. After 2 days, worms were washed and mechanically disrupted in M9 containing 1 % Triton X-100 and a series of dilutions were plated on LB (OP) or MRS agar (LAB). Each data point represents the mean cfu from triplicate samples ± standard errors. *Bars with different superscripts (a, b) differ significantly (P<0.05)*
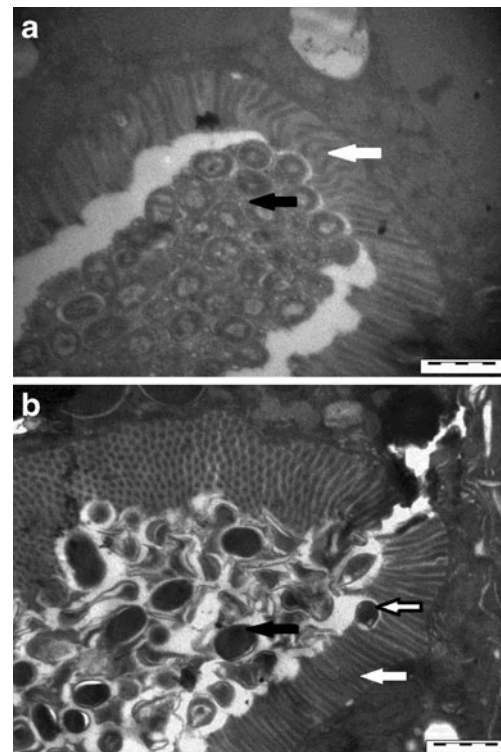

Fig. 3 TEM images of N2 worms showing **a** OP or **b** LS cells present within the intestinal track. Synchronized N2 L4 worms were moved from UV-killed OP plates to live OP and LS plates. After 7 days, worms were fixed and prepared for TEM. Images of the intestine show OP and LS being digested; *black arrow*: bacterial cells; *white arrow*: microvilli; *hollow arrow*: bacterial cell being digested (*scale bar* equals 1 μm)

(*P*<0.001) increased the mean lifespan, suggesting a different mechanism of action (Fig. 5a). With UV-killed bacteria, LR and PA again reduced the lifespan (Fig. 5b), while the increased lifespan observed with the live LS bacteria was reversed (Fig. 5b).

Tumor-like germ cell growth, DAF16 localization, and programmed cell death

When *gld-1⁻/⁻* worms were moved to LAB plates as 1-day-old adults, the growth of tumor-like germ cells was significantly inhibited (Fig. 6). The results were similar when UV-killed plates were used (data not shown). The result of tumor-like growth inhibition was also demonstrated by a survival experiment where *gld-1⁻/⁻* worms lived significantly longer on LAB compared to OP (Fig. 7).

We used transgenic worms expressing DAF16 fused to GFP to examine whether the presence of LAB could have an effect on DAF16 localization. Under normal conditions, GFP fluorescence is visible in the intestinal nuclei and cytoplasm. Stress (such as heat) causes DAF16 to localize in the nuclei that become much more
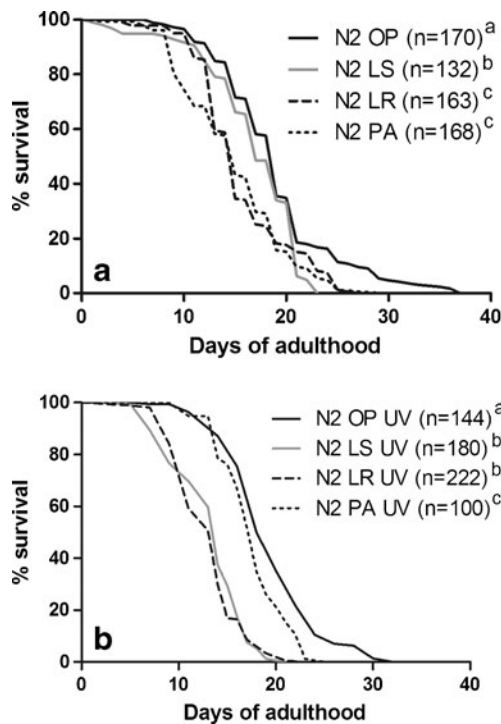
Fig. 4 Lifespan of N2 worms fed **a** live and **b** UV-killed LAB. One-day-old adult worms were transferred to NGM plates seeded with live or UV-killed cultures of OP or LAB. The plates were kept at 20 °C and the worms were transferred every 2 days to fresh plates. During transfer, worms were scored as dead if they failed to respond when touched with a pick. Worms that crawled off the agar or died as a result of internal hatching were removed from the experiment. Graph represents data from two independent experiments for each treatment. Mean lifespans of worms fed live OP, LS, LR, and PA were 19, 17, 15, and 15, respectively. Mean lifespans of worms fed UV-killed OP, LS, LR, and PA were 20, 14, 14, and 18, respectively. *Different superscripts* (*a–c*) indicate significant difference in mean lifespan (*P*<0.05)

Fig. 5 Lifespan of *daf-16* worms fed **a** live and **b** UV-killed LAB. One-day-old adult worms were transferred to NGM plates seeded with live or UV-killed cultures of OP or LAB. The plates were kept at 20 °C, and the worms were transferred every 2 days to fresh plates. During transfer, worms were scored as dead if they failed to respond when touched with a pick. Worms that crawled off the agar or died as a result of internal hatching were removed from the experiment. Graph represents data from two independent experiments for each treatment. Mean lifespans of *daf-16* worms fed live OP, LS, LR, and PA were 14, 17, 11, and 9, respectively. Mean lifespans of worms fed UV-killed OP, LS, LR, and PA were 17, 15, 13, and 15, respectively. *Different superscripts* (*a–c*) indicate significant difference in mean lifespan (*P*<0.05)

visible. It has also been shown that *P. aeruginosa* can inhibit nuclear localization of DAF16 (Evans et al. 2008). This can be seen following a heat shock. The results showed no difference in DAF16 localization between worms fed LAB and OP both under normal conditions and after heat stress (2 h at 35 °C and 16 h recovery) (Fig. 8).

We then examined whether LAB can increase corpse cells in N2 worms, *ced-4* mutants that lack programmed cell death (PCD) activity (Aballay and Ausubel 2001), and *gld-1* worms. The results showed no visible difference between the LAB plates and the control (OP) for all three worm strains (data not shown).

Immune response

To investigate whether LAB induce an immune response in *C. elegans*, we examined the expression of genes *lys-7*, *lys-8*, *thn-2*, and *sod-3*. When 1-day-old adult N2
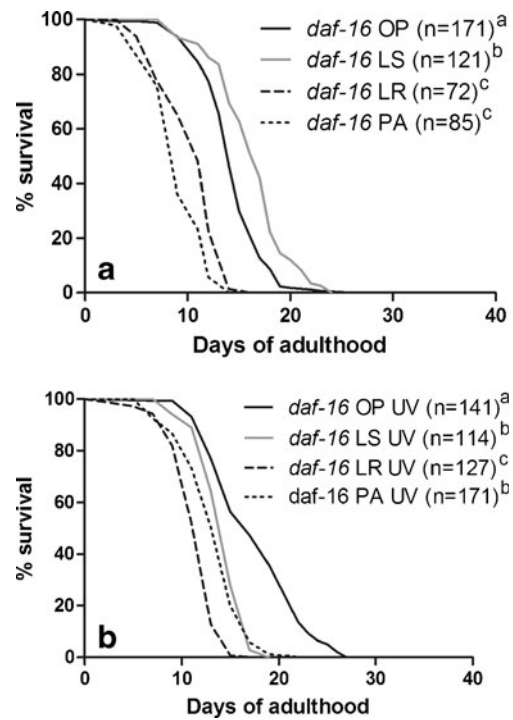
worms were left for 24 h on live LS plates, the expression levels of *lys-7* significantly (*P*<0.05) dropped (compared to live OP control) and the expression of *thn-2* increased approximately sevenfold (*P*<0.05) (Fig. 9a). On LR plates, expression of both *lys-7* and *thn-2* significantly increased (*P*<0.05 and *P*<0.01, respectively) with *thn-2* showing an 11-fold increase compared to the control (Fig. 9b). On PA plates, *lys-7* expression did not significantly (*P*>0.05) change, while *thn-2* transcript levels were nearly seven times higher (*P*<0.05) (Fig. 9c). No significant (*P*>0.05) change in expression levels of *lys-8* or *sod-3* were seen for any of the LAB plates (Fig. 9a–c). These results indicate that despite the fact that exposure to the LAB did not appear to cause nuclear localization of DAF16, it did induce an immune response involving *thn-2* and/or *lys-7*. As all four genes are targets of DAF16, the upregulation of only *lys-7* and *thn-2*, without nuclear localization of DAF16, suggests that pathways other than the insulin/insulin-like signaling are involved.

Fig. 6 DAPI stained *gld-1* worms fed **a** OP, **b** LS, **c** LR, or **d** PA. One-day-old adult *gld-1⁻/⁻* worms were transferred to NGM plates seeded with live lawns of OP or LAB. Three days later, worms were methanol-fixed and stained with DAPI. Confocal images show whole worms with stained nuclei of germ cells visible within the gonads (densely stained areas that fill the body). A reduced number of stained nuclei in the LAB samples compared to the OP sample where the images become saturated. Representative images of four independent experiments are shown (*scale bar* equals 50 μm)
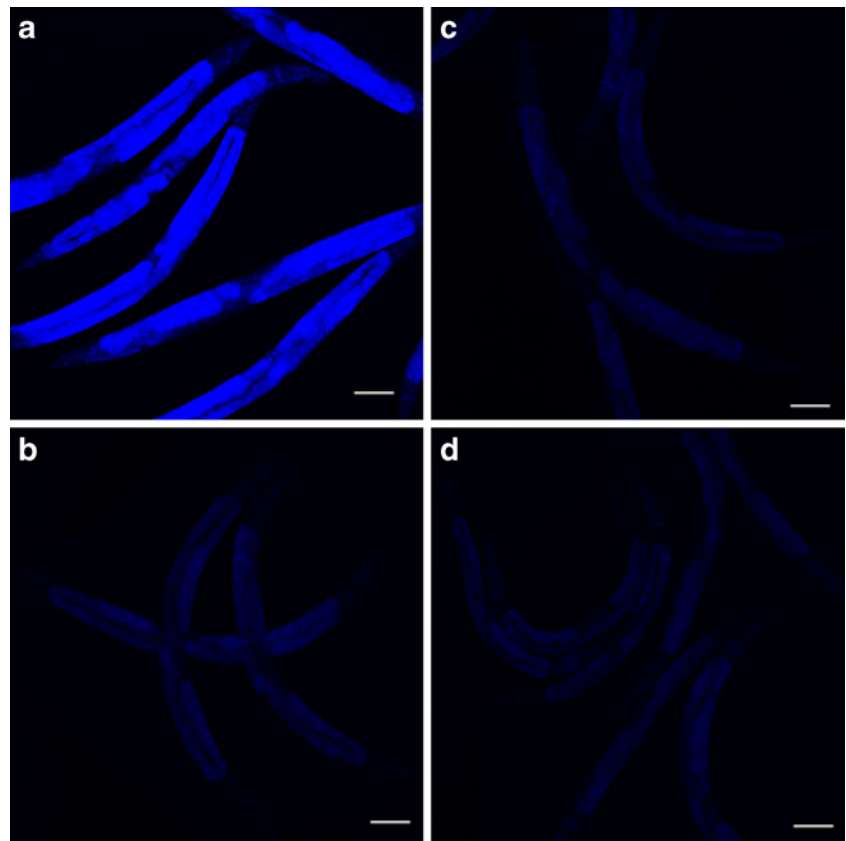
Discussion

The first evidence that LAB can be harmful to nematodes

Our results showed that the LAB inhibited the reproduction, growth, and development of *C. elegans*. For
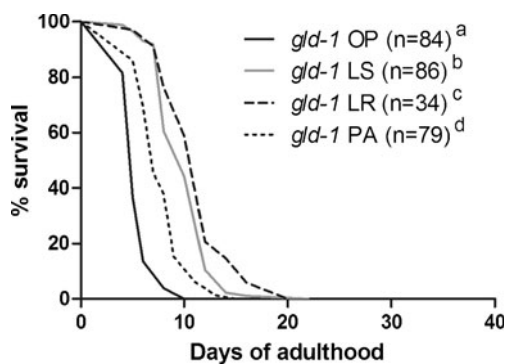

Fig. 7 Lifespan of *gld-1* worms fed OP or LAB. One-day-old adult *gld-1⁻/⁻* worms were transferred to NGM plates seeded with live lawns of OP or LAB. The plates were kept at 20 °C, and the worms were transferred daily to fresh plates. Worms were scored as dead when they burst and failed to move when touched with a pick. Mean lifespans of worms fed OP, LS, LR, and PA were 5, 10, 12, and 7, respectively. *Different superscripts* (*a–d*) indicate significant difference in mean lifespan ($P<0.05$)

this reason, the longevity experiments began using 1-day-old adults. Wild-type N2 worms, fed LAB, showed a significantly reduced lifespan (Fig. 4). This result was unexpected given that other LAB tested so far have been shown to have a beneficial effect on longevity (Ikeda et al. 2007). Generally, in *C. elegans*, dietary restriction or starvation extends lifespan (Houthoofd et al. 2007; Kaeberlein et al. 2006; Sutphin and Kaeberlein 2008). Therefore, a reduced lifespan should not reflect a lack in nutritional value. One factor that reduces longevity is the packing of proliferating bacteria within the intestine; feeding UV-killed OP reduces this effect (Garigan et al. 2002). For this reason, experiments using UV-killed bacteria were also conducted in this study. The results showed that UV-killed LS and LR further reduced the worm's lifespan while UV-killed PA did not significantly ($P>0.05$) affect it (Fig. 4b). This shows that live bacterial activity was not the cause of death, at least for LS and LR. Instead, the presence of live bacteria had a protective effect against the factor(s) that reduced the worm's lifespan.

To our knowledge, this is the first time LAB have been shown to have a negative effect on nematodes. Although this was not expected, given the results of other researchers, it could be suggested that the effect of LAB on *C. elegans* may differ significantly from strain to strain.

Fig. 8 Transgenic worms expressing DAF16:GFP fed **a** OP or **b** LS under normal conditions and **c** OP or **d** LS 16 h after heat stress (2 h at 35 °C). Fluorescence is seen in both nuclei (discs) and cytoplasm (background) under normal conditions for OP and LS (*small dots* are a result of intestinal autofluorescence). After heat shock, nuclear localization of DAF16 is visible in OP- and LS-fed worms (only nuclei and intestinal autofluorescence visible). Images are representative of at least three independent experiments. Results were the same for all LAB strains (*scale bar* equals 50 μm)
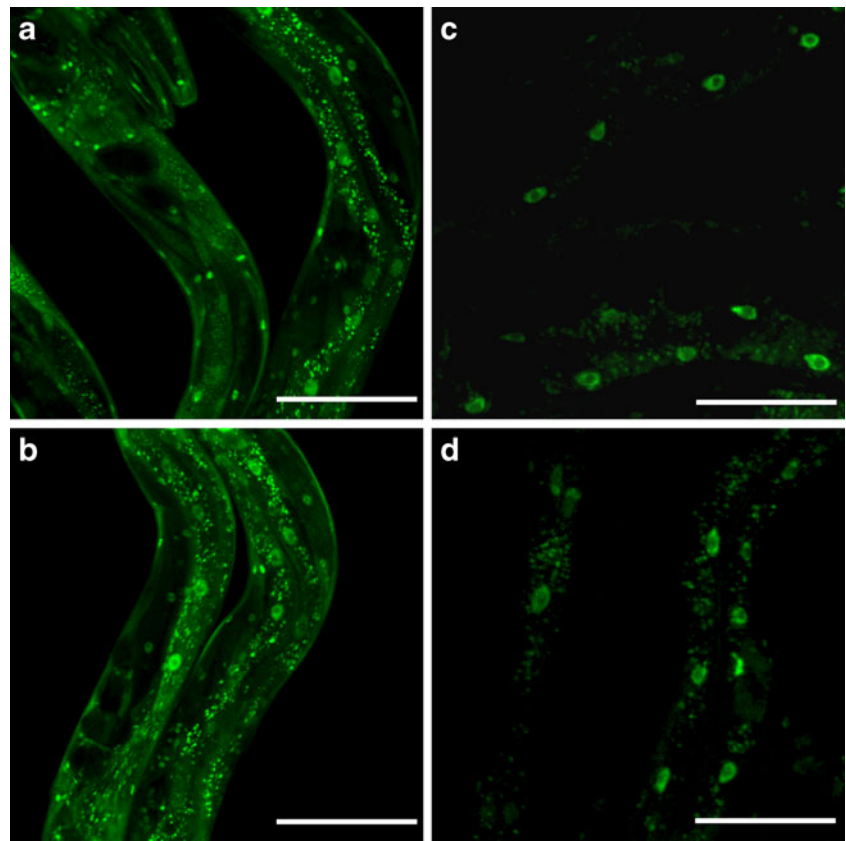
Ikeda et al. (2007) also observed that adult worms moved to LAB plates lagged slightly in growth for the first 3 days; however, after 4 days, they achieved the same body size as those fed OP. The LAB strains in this study did not allow the worms to grow as much as OP did even after 11 days (Fig. 1). As LAB produce bacteriocins effective against other microbes, the possibility that such a compound, produced by the specific strains used, may be responsible for this effect on *C. elegans*, cannot be excluded.

Effect of LAB may be the result of competing mechanisms

To further examine the effect of the LAB strains on the worm's lifespan, we also used the *daf-16(mu86)* mutant

Fig. 9 Results from real-time RT-PCR showing transcriptional response of *lys-7*, *lys-8*, *thn-2*, and *sod-3* to LAB. One-day-old adult N2 worms were left for 24 h on live OP or **a** LS, **b** LR, and **c** PA plates, and total RNA was isolated and reverse-transcribed to cDNA. Expression levels are relative to *ama-1* and the control treatment (OP) ± standard errors (*P<0.05; **P<0.01)
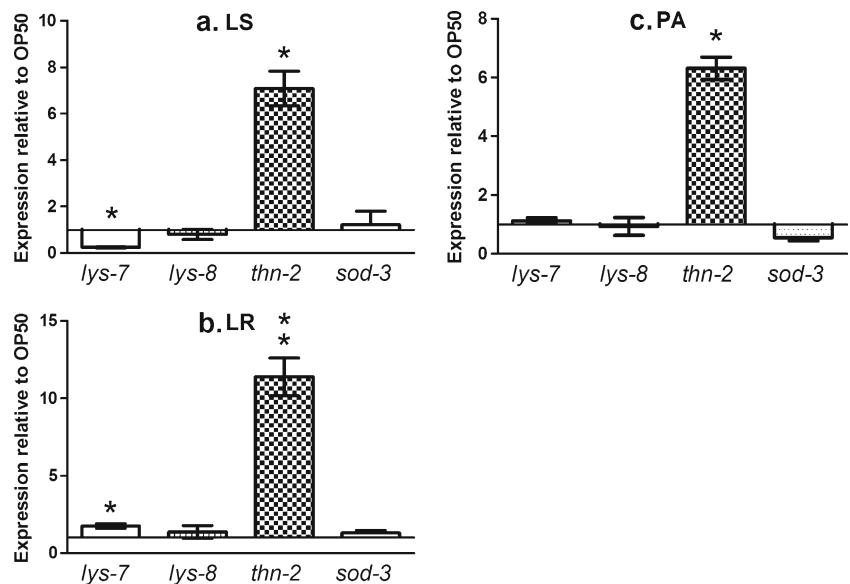
strain. DAF16 is a FOXO family transcription factor that is part of the insulin/insulin-like growth factor 1 signaling mechanism. The lack of DAF16 localization in the nucleus results in downregulation of a number of target genes involved in stress and immune responses. As such, *daf-16* mutants are short-lived and sensitive to pathogens or stress (Hsu et al. 2003). The fact that live LS counteracts the negative effect of UV-killed LS and that this is much more obvious in *daf-16* worms is interesting. In fact, *daf-16* worms fed LS lived as long as N2 worms fed LS (Figs. 4 and 5). One possible explanation for this may be that the reduced growth of worms fed LS results in less oxidative damage. This in turn has a greater effect on *daf-16* mutants that are more sensitive to such stress. However, this does not explain why the same effect is not observed with LR and PA. The presence of certain compounds may also be the cause of this effect. Such compounds have been found in royal jelly (Honda et al. 2011). Their presence extended the lifespan of both N2 and *daf-16* worms though the exact mechanism behind this remains unclear. It is possible that intermediate metabolites or rapidly degradable compounds found in LS are only present in sufficient concentrations in live cells. Overall, it seems likely that the effect of LAB is not simply due to bacteriocins and may be the result of competing mechanisms.

LAB inhibited tumor-like germ cell growth but did not affect DAF16 localization or increase corpse cells

As LAB are presumed to have antitumor effects, we used worm strain JK1466 to isolate *gld-1⁻/⁻* individuals and examine the effect of LAB on tumor-like growth. *gld-1* is a tumor suppressor the lack of which causes germ cells that enter the meiotic pathway normally to then exit pachytene and return to the mitotic cycle, leading to continuous proliferation of cells that fill the body of the worm and finally kill it (Francis et al. 1995). This strain has been used to study the effectiveness of antitumor drugs (Wilson et al. 2008). When *gld-1⁻/⁻* worms were moved to LAB plates as 1-day-old adults the growth of tumor-like germ cells was significantly inhibited (Fig. 6). This effect was expected given that the worm's growth was also inhibited. The result of tumor-like growth inhibition was also demonstrated by a survival experiment where *gld-1⁻/⁻* worms lived significantly longer on LAB compared to OP (Fig. 7).

Localization of DAF16 in the nucleus, as part of an immune response, can be induced or inhibited by pathogens (Evans et al. 2008; Singh and Aballay 2009). We therefore used transgenic worms expressing DAF16 fused to GFP to examine whether the presence of LAB could have such an effect. The results showed no difference between worms fed LAB and OP both under normal conditions and after heat stress (DAF16 localizes in the nucleus) (data not shown).

In *C. elegans*, another response to pathogens or stress can be the activation of PCD (Aballay and Ausubel 2001). LR has been shown to induce apoptosis in myeloid leukemia-derived cells induced by tumor necrosis factor (Iyer et al. 2008). We examined whether LAB can increase corpse cells in N2 worms, *ced-4* mutants that lack PCD activity (Aballay and Ausubel 2001), and *gld-1* worms where we previously demonstrated a significant reduction in tumor-like germ cell proliferation (Fig. 6). The results showed no visible difference between the LAB plates and the control (OP) for all three worm strains (data not shown).

LAB strains induce a DAF16-independent immune response

To investigate whether LAB induce an immune response in *C. elegans*, we examined the expression of genes *lys-7*, *lys-8*, *thn-2*, and *sod-3*. *lys-7* and *lys-8* are expressed downstream of *daf-16* and encode lysozymes. Expression levels of *lys-7* and *lys-8* have been shown to be upregulated in response to infection by the Gram-negative bacterium *Serratia marcescens* (Mallo et al. 2002). *thn-2* encodes a member of the thaumatin family, an immune effector the downregulation of which results in increased sensitivity to *P. aeruginosa* infection (Evans et al. 2008). *thn-2* expression is upregulated in *daf-2* mutants suggesting that it is also a target of DAF16 (Murphy et al. 2003). *sod-3*, another target of DAF16, encodes an iron/manganese superoxide dismutase that protects against oxidative stress and whose expression levels are correlated with the worm's remaining lifespan (Sánchez-Blanco and Kim 2011).

The response to the three LAB strains seen here shows that despite the fact that exposure to the LAB did not appear to cause nuclear localization of DAF16, it did induce an immune response involving *thn-2* and/or *lys-7*. As all four genes are targets of DAF16, the upregulation of only *lys-7* and *thn-2*, without nuclear localization of DAF16, suggests that pathways other than the insulin/insulin-like signaling are involved. This response also conforms with the strain-specific immune responses described by Alper et al. (2007).

Inhibitory effect on tumor-like growth is confirmed, possible use as model system

The inhibition of tumor-like germ cell growth demonstrated here suggests that the worm could be useful for examining the mechanism by which LAB can exert anticancer activity. Though this has been the subject of many studies, there is mostly indirect evidence so far that LAB can protect against or suppress cancer (Kumar et al. 2010). While the overall effect of the examined LAB strains on *C. elegans* appeared to be negative, there is evidence here that they can also have a positive effect (such as the extended lifespan of *gld-1* and *daf-16* worms fed LS). Further investigation of such

mechanisms in *C. elegans* may lead to the identification and isolation of compounds produced by LAB with therapeutic or antitumor activity.

LAB may be effective against parasitic nematodes

Our work showed that not only did the LS, LR, and PA cultures fail to extend the worm's lifespan, but they also significantly reduced it while also inhibiting egg hatching and larval development (Figs. 1 and 4). *C. elegans* has also been used as a model for parasitic nematodes (Gilleard 2004). Even though there is a significant genetic variation among these species, the evidence here should justify examining the possibility of using LAB as anthelminthics. There are numerous studies concerning the use of such cultures in farm animals to fight bacterial and viral infections though little attention has been given to their effectiveness against parasites (Gill 2003; Glass et al. 2004; Tierney et al. 2004). A recent review by Travers et al. (2011) describes how this subject is gaining interest along with the need to better describe the molecular mechanisms involved.

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