Lactic acid bacteria that activate immune gene expression in the *Caenorhabditis elegans* can antagonize the *Campylobacter jejuni* infection in nematodes, chickens and mice

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Abstract

Background

Campylobacter jejuni is the major microbacillary pathogen responsible for human coloenteritis. Lactic acid bacteria have been shown to protect against Campylobacter infection. But LAB that showed a good ability to inhibit the growth of C. jejuni in vitro are less effective in antagonising C. jejuni in animals and animal models have the disadvantages of high cost, a long cycle, cumbersome operation and insignificant immune response indicators. Caenorhabditis elegans is increasingly used to screen probiotics for anti-pathogenic property. However, no research on the use of C. elegans to screen for probiotic candidates antagonistic to C. jejuni has been conducted to date.

Results

This study established a Caenorhabditis elegans life-span model enabling preselection of lactic acid bacteria to counter Campylobacter jejuni infection. A potential protective mechanism of the LAB was identified. 9 strains of distinct LAB species offered the high level of protection for Caenorhabditis elegans. Many of the LAB strains with a high protection rate reduced Campylobacter jejuni load in nematodes. The transcription of antibacterial peptide genes, MAPK and Daf-16 signalling pathway related genes was elevated by the LAB isolates with a high protection rate. The reliability of the life-span model of Caenorhabditis elegans was verified using Campylobacter jejuni-infected mice and chicken model respectively.

Conclusions

These results showed that LAB vary in their ability to protect Caenorhabditis elegans against Campylobacter jejuni, and Caenorhabditis elegans provides a reliable model enabling researchers to screen for LAB antagonistic to Campylobacter jejuni on a large scale.

Background

Infection by pathogenic Campylobacter may result in symptoms such as bloody diarrhoea, abdominal pain and fever. In many countries, Campylobacter species is the major microbacillary pathogen responsible for human coloenteritis[1]. In under developed countries, diarrhoeic disease is 10 times more likely to result from Campylobacter infection than from infection with Escherichia coli O157: H, Shigella species or Salmonella species[2, 3]. Some peripheral neuropathies, such as Guillain-Barré syndrome and Miller Fieher syndrome, are long-term consequences of Campylobacter infection[2]. Campylobacter jejuni (C. jejuni) is also one of the primary reasons for microbacillary food-borne disease in some developed countries[3]. To date, all therapies for Campylobacter infection involved antibiotics, especially in poultry industry. There is an urgency to develop alternative approaches due to a gradual increase in antibiotic-resistant Campylobacter[4]. It is also necessary to illustrate the mechanisms underlying the functions of such alternatives to support their development and implementation.
Research has increasingly shown that lactic acid bacteria (LAB) colonise the human gastrointestinal tract and play a vital role in maintaining intestinal function and well being of the host. Among the beneficial effects of LAB are bacteriostatic activities targeting pathogens such as *Escherichia coli* (*E. coli*), *Salmonella* and *Listeria monocytogenes*\[5, 6\]. LAB have been shown to protect against *Campylobacter* infection. Nishiyama reported that LAB inhibited *Campylobacter* and colonisation of this pathogen was reduced by *Lactobacillus gasseri* SBT2055 isolated from healthy chicken\[7\]. Another study reported a decrease in *C. jejuni* invasion in the gut of turkey poults after treatment with *Lactobacillus salivarius* NRRL B-30514, suggesting that competitive exclusion can play a role\[8\]. Wagner et al. conducted a simulation experiment with immunodeficient and immunocompetent mice, they found that bifidobacteria and lactobacilli can increase colonisation resistance. Specifically, the results showed that bifidobacteria and lactobacilli can resist *C. jejuni* enteric persistence in the human gut\[9\]. To date, studies of antagonism against *C. jejuni* by LAB have mainly been conducted *in vitro* or *in vivo*. Unfortunately, some of the strains of LAB that showed a good ability to inhibit the growth of *C. jejuni* *in vitro* are less effective in antagonising *C. jejuni* in animals. Whether poultry or mice, all animal models have the disadvantages of high cost, a long cycle, cumbersome operation and insignificant immune response indicators, and do not permit rapid large-scale screening for LAB capable of effective antagonising *C. jejuni*.

The small, free-living (non-parasitic) soil worm *Caenorhabditis elegans* (*C. elegans*) has been widely used in biological studies as a model *in vivo*, due to its short generation time, diminutive form and clear genetic background. To date, *C. elegans* has been used to study a series of pathogenic microorganisms, such as *Salmonella enterica*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*\[10–14\]. *C. elegans* is also increasingly used to screen for antimicrobials and probiotics\[15\]. In addition, institutional studies of the effects of LAB can be processed using *C. elegans*. However, no research on the use of *C. elegans* to screen for probiotic candidates antagonistic to *C. jejuni* has been conducted to date. Moreover, the molecular mechanisms underlying the protective mechanism of LAB have yet to be established. In this study, a *C. elegans* life-span experimental model was developed to investigate the response of nematodes to *C. jejuni* infection, enabling rapid evaluation of the protective effects of LAB and understanding of microbe-host interactions. A *Toxoplasma gondii* (*T. gondii*) induced acute ileitis model abrogating the colonisation resistance in mice and chicken to *C. jejuni* infection was also used to validate the *C. elegans* model\[16\].

**Results**

*C. jejuni* intake shortened life-span of *C. elegans*

The harm of foodborne pathogens to *C. elegans* was usually reflected in the life-span of the nematodes. A *C. elegans* life-span experimental model was used to measure the responses of the worm to *C. jejuni* infection (Fig. 2A). *E. coli* OP50 was used as the food that normally sustains nematodes on reaching the L4 stage. As shown in Fig. 2B, *C. jejuni* killed about 50% of the worms within 5 days of their transferral to the lawn of the pathogen (C/day0), whereas 80% of the worms fed OP50 were still alive after 8 days (E/day0). All the nematodes in the E/day0 group died within 21 days; the equivalent period in the C/day0 group was 13 days. When the L4 stage nematodes were fed OP50 for 3 days before *C. jejuni* treatment (E/day0 + C/day3), they proved more resistant to *C. jejuni*. nearly 50% of these worms were still alive on day 10. However, their life-span was shorter than that of the worms fed OP50 only (E/day0). In addition, the initial number of *C. jejuni* cells recovered from the worms in the E/day0 + C/day3 group was smaller than that of the *C. jejuni* cells recovered
from the worms in the C/day0 group (Fig. S1). This indicated that *C. elegans* at day 0 of the L4 stage was most sensitive to *C. jejuni* infection. Therefore, worms at day 3 of the L4 stage were chosen for the life-span assay due to the moderate life-span of the worm, pathogenicity of *C. jejuni* at this time point and available time space for LAB intervention.

The body size of *C. elegans* directly reflects the growth and development of nematodes which is closely related to their energy intake. In addition to pathogenicity, pathogens may also affect the life-span of nematodes due to their inability to be metabolized by *C. elegans*. To determine whether worms’ life-span shorten by *C. jejuni* was due to caloric intake or pathogenicity of *C. jejuni*, the body size of the nematodes fed *C. jejuni* was compared with that of the worms in the E/day0 group (Fig. 2C). The (E + C)/day0 group nematodes were fed equal amounts of *E. coli* OP50 and *C. jejuni* concurrently from day 0 of L4 stage. For 8 days, the nematodes in the (E + C)/day0 group and the E/day0 + C/day3 group were nearly the same size as those in the E/day0 group, which indicated that the three groups had almost the same caloric intake during this period. Furthermore, although the intestinal load of *C. jejuni* in the nematodes in the E/day0 + C/day3 group was much larger than the load in the (E + C)/day0 group, the load of *C. jejuni* in each of the two groups remained stable (Fig. 2D), which indicated that the nematodes showed no preference for ingesting *C. jejuni* versus *E. coli*. Therefore, substituting *C. jejuni* for *E. coli* did not lead to fasting, which may have resulted in worm death.

**Some LAB strains prolonged the life-span of** *C. elegans* **treated with** *C. jejuni*

A prolonged nematodes’ life-span after infection is a common indicator of antibacterial ability in *C. elegans*. To enable rapid evaluation of the defensive effects of LAB, forty-four LAB were assessed on their ability to protect *C. elegans* from *C. jejuni* infection mediated death. On day 13, *C. elegans* fed only *E. coli* OP50 displayed 50% survival (LD50) whereas the survival of nematodes fed only *C. jejuni* was only 20%. As shown in Fig. 3, S2 and Table 1, S2, the LAB isolates varied in their ability to protect the live worms, with survival rates ranging from 15–47%. Among tested isolates, Z5, 13M2, N9, L103, G20, 1132 and 13 − 7 provided high levels of protection (each producing an approximately 40% worm survival rate), whilst 422, B and Z6 did not show significant protection.

**Some LAB strains decreased the *C. jejuni* load in the intestine of** *C. elegans*

Diminishing pathogenic bacterial colonisation in the intestinal tract is probably one of underlying mechanisms of LAB in prolonging nematodes’ life-span. Also, the live LAB cells colonized in the *C. elegans* intestine played a role in decreasing the number of pathogenic bacteria. To investigate whether association exist between the life-span of *C. elegans* and the load of different bacteria in nematodes’ intestine, the number of LAB, *C. jejuni* and *E. coli* OP50 in intestine were counted. Twenty-six LAB strains which showed various levels of protection were evaluated on their ability to persist in the worm intestine from day 2 to day 6 (Fig. 4 and S3). The loads of LAB strains such as Z5, 427, N34 and X13, which showed a strong ability to protect *C. elegans* against death (37.52%-40.36% survival rate) exceeded log10⁵ CFU/worm during the assay. In contrast, the loads of B, G14, ZX7 and Z7 were found to be low (log10⁴ CFU/worm), also showed low levels of nematode protection in the life-span assay. The *C. jejuni* loads in these worms from day 2 to day 6 were also checked. Similarly, different LAB strains were associated with different levels of *C. jejuni* loads (log10⁳.⁵–⁴.⁵ CFU/worm) in the nematode intestine during the assay (Fig. 5 and S4). The *C. jejuni* load in *C. elegans* treated with Z5, X13, 13 − 7, 427, G20 and N9 (offering high levels of protection in terms of worm life-span, 38.02%-43.28% survival rate) was almost
1.5 orders of magnitude smaller than that in the control group (E/day0 + C/day3, log10^{4.5}), whereas B, Z6, LGG and 422 (offering low levels of protection in terms of worm life-span) did not significantly decrease the *C. jejuni* load. The results of correlation analysis showed that the LAB load was highly positively correlated and conversely the *C. jejuni* load was highly negatively correlated with the survival of *C. elegans*. In addition, the load of LAB was moderately correlated with the load of *C. jejuni* in *C. elegans* (Table 2). It is worth noting that, there were no obvious differences in OP50 load among the E/day0 + C/day3 group and other LAB intervention groups (log10^{1.2–1.5} CFU/worm) on day 6 (Fig. S5).

Longevity effects of LAB on *C. elegans* treated with *C. jejuni* were not due to caloric reduction

The body size of *C. elegans* directly reflects the growth and development of nematodes, which is closely related to their energy intake. Except the body size, pharyngeal pumping represents the feeding capacity of *C. elegans* and is a key index for the physiological activities. Also, it is an important parameter to evaluate the toxicity of drugs in *C. elegans*. To determine whether the longevity effects of LAB were the result of caloric reduction, the body size (Fig. 6A, B and Fig. S6) and pharynx pumping (Fig. 6C and Fig. S7) of nematodes fed 26 LAB strains and *C. jejuni* (L/day0 + C/day3 groups) was compared with that of nematodes in the E/day0 + C/day3 group. The worms in the E/day0 + C/day3 group and those in the LAB intervention groups showed little differences in body size, with the exceptions of the worms treated with N34, 675, 427 and 676, which were smaller. However, the N34, 675, 427 and 676 strains had varying protective effects (37.52%, 22.67%, 38.02% and 27.05% survival rate on day 13, Table S2). These were not the top survival rates in the life-span assay. The same situation also appeared in the determination of nematodes’ pharynx pumping. The pharynx pumping of worms was at the range of 50 to 58 per 30 s in the E/day0 + C/day3 group and most LAB intervention groups except for N34, 675, 427 and 676 treated groups. There were no significant differences in body size and number of pharynx pumping of nematodes treated by these LAB which showed varied longevity effects on *C. elegans* treated with *C. jejuni*.

Influence on *C. jejuni* growth by co-culturing *E. coli* OP50

To investigate whether the substance produced by *E. coli* OP50 such as certain bacteriocins would kill *C. jejuni*, the viability of *C. jejuni* cultured with or without live *E. coli* OP50 was measured. As showed in Fig. S8, the number of *C. jejuni* elevated in both tests after a 24-h incubation did not show significant difference. Furthermore, the pathogen was uninfluenced after the live *E. coli* OP50 were added to the growing *C. jejuni* after 48 h of incubation.

Some LAB strains upregulated immune gene transcription to protect *C. elegans* against *C. jejuni* infection

Regulation on the host’s immune system through pivotal signalling pathways is also an underlying mechanism by LAB on prolonging nematodes’ life-span against pathogen infection. To determine the mechanism by which LAB protected *C. elegans* against *C. jejuni* infection, the transcription levels of the 14 immune genes (*tir-1, nsy-1, sek-1, pmk-1, spp-1, clec-85, abf-2, clec-60, lys-7, daf-16, age-1, dbl-1, skn-1 and bar-1*) of *C. elegans* on day 6 were compared between the E/day0, E/day0 + C/day3 and L/day0 + C/day3 groups. As shown in Fig. 7 and S9, the transcription of *tir-1, pmk-1* and *bar-1* (respectively MAPK signalling pathway genes and an antioxidant gene) was to some extent increased when nematodes were infected with *C. jejuni*. In addition, slight increases
were observed in some of the defence immune genes of *C. elegans* infected with *C. jejuni*, such as *daf-16* and *age-1* (Daf-16 signalling pathway genes) and *dbl-1* (a TGF-β signalling pathway gene).

The 14 immune genes of *C. elegans* were also examined after treatment with 11 LAB strains, which showed variation in the protection against *C. jejuni*-induced worm death (Fig. 7, S9 and Table S3). It was found that the immune genes of *C. elegans* did not change significantly after LAB intervention without *C. jejuni* infection, which indicated that LAB strains were safe to healthy host (Table S3). As shown in Fig. 7 and S9, when nematodes were treated with LAB offering low levels of protection for survival, the transcription levels of their defence genes were almost identical to those of nematodes infected by *C. jejuni* alone. For these LAB strains, such as PC-T7 and B, only some genes (such as *tir-1* and *skn-1*) were transcribed at a slightly higher rate than their counterparts in the group infected with *C. jejuni* alone, and the expression of *spp-1* and *bar-1* was even inferior to that in the control group. The transcription levels of some genes (such as *tir-1*, *pmk-1* and *bar-1*) were much lower in some of the groups treated with poorly protective LAB (422, G14) than in the group infected with *C. jejuni* only. On the contrary, for those LAB strains, which protected the nematodes against *C. jejuni*-induced death in the life-span assay, the transcription levels of genes assayed were increased considerably on day 6. For example, treatment with 13–7, N9 and Z5 significantly enhanced the transcription of the MAPK signalling pathway genes *nsy-1*, *sek-1* and *pmk-1*, the antioxidant gene *skn-1* and the Daf-16 signal pathway genes *age-1* and *daf-16*. The levels of transcription of the antibacterial peptide genes *spp-1*, *clec-85* and *lys-7* in these LAB intervention groups were 3–4 times higher than those in the group infected with *C. jejuni* alone. These data indicated that the ability of LAB to protect nematodes against *C. jejuni*-induced death was correlated with their influence on the levels of transcription of immune genes.

**LAB strains which prolonged the life-span of C. elegans decreased the C. jejuni load in mice with T. gondii induced acute ileitis**

LAB that showed protection of nematodes against bacterial infection might not applied to mammals due to obvious differences between the two organisms. To investigate whether the LAB screened from *C. elegans* model had the same effects in mammal, 11 LAB strains with different effects on *C. elegans* immune gene transcription were further investigated, to determine their capabilities to decrease the load of *C. jejuni* in mice. Seven days after *T. gondii* infection, the mice developed acute ileitis and likely to die, so the *C. jejuni* loads were checked on day 5. The load of *C. jejuni* in the faeces of mice in the *C. jejuni* infected group reached $10^8$ CFU/g (Fig. 8A). LAB strains 13–7, Z5 and G20, which had already shown an outstanding ability to protect the nematodes, also exerted superior suppressive effect on *C. jejuni* load (less than $10^6$ CFU/g faeces) in the mouse intestinal tract. Strains 422 and G14, which showed poor protective effects in terms of worm life-span, correspondingly played an inconspicuous role in suppressing *C. jejuni* load (around $10^8$ CFU/g faeces). In addition, PC-T7 and Z6, which showed slight depressive effects on *C. jejuni* load ($10^7$–$10^8$ CFU/g faeces) had previously offered moderate and low protection, respectively, to the worms. Meanwhile, N9 and 430, which had moderate depressive effects on *C. jejuni* load ($10^6$–$10^7$ CFU/g faeces), had previously offered the worms high and moderate levels of protection, respectively. Therefore, the effects of all the 11 LAB strains apart from B and 427 were consistent across the *C. elegans* and mice samples.

Correlation analysis was conducted to examine the relationship between the ability of the 11 LAB strains to clear *C. jejuni* in mice and the survival rate of *C. jejuni*-infected nematodes. The relative index $R^2$, which reached
0.79093, indicated that the *C. jejuni*-antagonistic activity of LAB in *C. elegans* was significantly co-related to their *C. jejuni*-antagonistic activity in mice (Fig. 8B).

**LAB strains which prolonged the life-span of *C. elegans* decreased the *C. jejuni* load in chicken**

Outbreaks of campylobacteriosis can occur if humans ingest undercooked poultry contaminated by the *C. jejuni*. LAB applied in fodder could reduce *Campylobacter* colonization in poultry and stop the disease outbreak at its source. To investigate whether the LAB screened from *C. elegans* had the same effects in poultry, 11 LAB strains with different effects on *C. elegans* immune gene transcription were investigated to determine their abilities to clear *C. jejuni* in chicken. The inhibitory effect of LABs on *C. jejuni* colonization in chicks' cecum was examined. Approximately 24 h after hatching, chicks were inoculated orally with *C. jejuni*, and then LAB was administered daily for two weeks. CFU of *C. jejuni* in chicken cecum in all groups were evaluated on day 23. The average value of *C. jejuni* increased to $10^8$ CFU/g cecal content in the *C. jejuni* infected group (Fig. 9A). Z5 and 427, exerted most significant suppressive effects on *C. jejuni* colonisation in the chicken cecum, which resulted in *C. jejuni* loads fell to below $10^4$ CFU/g cecal content in these two groups. Correspondingly, Z5 had demonstrated an outstanding ability to protect the nematodes and decrease *C. jejuni* load in the mouse intestinal tract, while the antagonizing ability by 427 in mice was reversed. Meanwhile, 430, B and G14 showed poor abilities to clear *C. jejuni* in chicken cecum. The *C. jejuni* loads in the last three groups were higher than $10^7$ CFU/g cecal content. The effects of these 3 strains on protecting the nematodes were similar to that of elimination of *C. jejuni* in chicken. However, 430 and B exhibited moderate strength of *C. jejuni* antagonization in mice, which was different from their performance in the chicken. In addition, 13 – 7, N9, G20, PC-T7, 422 and Z6 showed moderate scavenging activity on *C. jejuni* in the chicken cecal contents with the load of *C. jejuni* at the range from $10^{4.8}$ to $10^{5.5}$ CFU/g faeces. It is worth noting that a few strains (422 and Z6) showed a certain degree of inconsistency in the *C. jejuni* antagonism in different models.

Correlation analysis was conducted to examine the relationship between the ability of the 11 LAB strains to clear *C. jejuni* in chicken and the survival rate of *C. jejuni*-infected nematodes. The relative index $R^2$, which reached 0.50071, indicated that the *C. jejuni*-antagonist activity of LAB in *C. elegans* was related to their *C. jejuni*-antagonistic activity in chicken (Fig. 9B).

**Discussion**

The model organism *C. elegans* has been treated with numerous pathogenic microorganisms from animals and humans to examine the relationships between pathogenic bacteria and their hosts[10–14]. In this study, a *C. elegans* life-span experimental model was first established to examine the response of the nematode to *C. jejuni* infection. Our experiments revealed that LAB strains varied in their ability to defend the nematodes against infection with *C. jejuni* NCTC 11168. Also, the antagonism effects of LAB isolates on *C. jejuni* in worms were verified in both mice and chickens. The worm life-span experimental model not only provided a useful way of screening for LAB candidates with the potential to mitigate *C. jejuni* infection, but also helped to explain the mechanism of the defence imparted by the LAB isolates. In addition, the findings indicated that LAB defended *C. elegans* not only by inhibiting *C. jejuni* colonisation in the intestine, but also by activating the nematodes' defence immune genes.
The natural physiological activities of nematodes are usually halted by the colonisation of invading pathogens[17–19]. Some LAB (including *L. rhamnosus*[20], *L. acidophilus*[21], *L. fermentum*[13], *L. gasseri* and *L. plantarum*[22]) have been reported to show high shielding efficiency to nematodes’ from pathogens and to diminish the risk of bacterial colonisation in the intestinal tract and prolonging life-span. The intestinal colonisation of *C. jejuni* may occur in humans or animals, and is often observed in poultry. The binding of fimbrial adhesins to the host’s intestinal tract is a condition for *C. jejuni* nosogenesis, which leads to diseases such as diarrhoea[23, 24]. Many investigators have showed that *C. jejuni* colonisation and growth in the host intestinal tract can be reduced by sectional strains of LAB[25, 26]. The current article confirmed that the level of *C. jejuni* in the nematode intestinal tract was affected differentially by LAB strains, which offered strong to weak levels of protection. The *C. jejuni* load in the nematodes was highly correlated with the nematodes’ life-span, which indicated that the inhibition of *C. jejuni* colonisation in the intestinal tract was one of the mechanisms through which LAB offered protection to the worms. As reported, some foodborne pathogens, such as *Salmonella typhimurium*[21] and enteroinvasive *Escherichia coli*[27], colonized in the nematode intestinal tract to form a persistent lethal infection. The colonized LAB in the intestine produce antibacterial products continuously, they also occupy the adhesion sites of pathogens, which jointly exert antibacterial effects. However, in this study, LAB load and *C. jejuni* load in *C. elegans* were only moderately correlated, indicating that some LAB may alleviate the damage caused by infected pathogens in other ways, such as through worms’ immune gene expression regulation.

In non-mammalian taxa, mice and some primates, calorie restriction is normally used to increase longevity and ease the consequences of aging[28–30]. For *C. elegans*, researchers investigated the caloric intake as well in *C. elegans* intervened by LAB through measurement of body size and pharyngeal pumping rate[10–14]. The growth curves obtained in this study for most of the LAB intervention groups did not support the assumption that LAB is a lower-calorie food than *E. coli*. The LAB-induced morphological and swallowing ability changes in the nematodes were not related to the life-span extension of *C. elegans* infected by *C. jejuni*. Therefore, the prolongation of worms’ life-span could not be attributed to calorie restriction by the substitution *E. coli* with LAB strains.

With the growth and reproduction of LAB in a host’s intestine, the host’s immune system is regulated through pivotal signalling pathways with LAB involvement[31]. The host’s immune response to pathogens may be induced by LAB[32]. LAB strains with protective efficacy, such as *L. rhamnosus* GG[33], *L. plantarum*[34] and *L. delbrueckii*[35], enhance the host immune defence against *E. coli, Salmonella typhimurium* and *Streptococcus pyogenes* infection by exciting the MAPK signalling pathway and through toll-like receptors. LAB may also defend the host by adjusting gene expression through cytokine and chemokine activity[36, 37]. Another possible mechanism is the enhancement of membrane barrier[38, 39]. *C. elegans* has two main signalling pathways, including the P38-MAPK and TGF-β signalling pathways. Its specific defence system, including antimicrobial responses, falls short of an adaptive immune system[40]. Nevertheless, the upregulation of *tir-1, nys-1, sek-1* and *pmk-1*, MAPK pathway genes enabling it to resist various microbial infections. The findings demonstrated the activation of the MAPK pathway through LAB intervention[31]. In this study, the LAB strains offering good levels of protection for *C. elegans* against *C. jejuni* infection, and significantly enhanced the transcription of the MAPK pathway genes. Besides, as a downstream molecule regulated by the MAPK signalling pathway, the forkhead family transcription factor DAF-16 has been shown to adjust genes to improve dauer formation in the larval phase of nematodes, and to increase resistance and longevity when
mature[41–43]. In this study, it is consistent with the above findings, suggesting that these LAB strains increase worms’ defence by upregulating DAF-16 via the MAPK signalling pathway and simultaneously prolonging the worms’ life-span.

In addition, the cluster of C-type lectins has been argued to mask bacterial attachment and enhance resistance to microbial infection[44]. The increased levels of clec-60, clec-85, spp-1 and abf-2 upon LAB pre-treatment in this study also testify to the ability of some LAB to protect nematodes against *C. jejuni* infection. Skn-1 and bar-1 may also play critical roles in defending the host and prolonging its survival. The increased level of *skn-1* in the pre-treated nematodes was due first to antioxidant defence and second to the worms’ improved survival[45–47]. Partly consistent with this claim, *skn-1* transcription in the current study was significantly enhanced by LAB strains protective of nematode life-span. However, the same enhancement was also observed in worms treated with B and PC-T7. The transcription of *bar-1*, which reflects a response to the stress caused by *C. jejuni* infection, was unaffected by LAB strains with protective effects but down-regulated by some un-protective LAB strains, such as B, 422, G14 and Z6. It was confirmed that un-protective LAB strains could not increase the expression of *skn-1* and *bar-1* to extend the life-span of nematodes.

Although the TGF-β pathway is a major defence system in *C. elegans*, *dbl-1* showed almost no obvious changes in transcription level across the LAB intervention groups, which indicated that this gene may not play an essential role in protecting against *C. jejuni* infection. These signalling pathways mentioned above which were highly conserved could be found among numerous homologus genes in mammals and played a pivotal role on growth promotion, acclimatization, resistance to pathogens and adaptation of external stress in *C. elegans*. Based on this, *C. elegans* were more widely used as the hosts of zoonotic pathogens by LAB intervention to study the change of virulence factors and antagonism to pathogenic mechanisms. But not limited to these, antibacterial substances, competition and repulsion, adhesion barrier and immunomodulatory from LAB also showed the characteristics of antagonizing pathogenic bacteria in host. It is worth noting that the immune genes of *C. elegans* without *C. jejuni* infection did not change significantly after LAB strains intervention. So the LAB strains did not make a significant effect on healthy body which means LAB is safe to healthy host. LAB only played a significant role when the nematodes received external infection.

As the growth temperature of *C. elegans* is much lower than the temperature in the human gut, and the microbiota of *C. elegans* is much simpler than that of a mammal, the reliability of this *C. elegans* life-span assay in testing the efficiency of LAB antagonism against *C. jejuni* colonisation in mammals should be confirmed. In addition, *C. jejuni* contamination is a serious problem in poultry production. The *C. jejuni*-infected chicken model has been widely used in screening of probiotics for feed with *C. jejuni* antagonism, which could be applied as antibiotic substitutes[16]. In mammal model, a few LAB strains in the current study yielded the opposite results in terms of the survival of *C. elegans* and the pathogen load in mice, which may be due to host species differences or to the interference of gut microbiota in mice. Nevertheless, the significant correlation between the ability of LAB to clear *C. jejuni* in mice and their ability to enhance the survival rate of *C. jejuni*-infected nematodes indicates that the life-span model of *C. elegans* infected with *C. jejuni* can to some extent be applied to *C. jejuni*-infected mammals. The short cycle of acute infection in mice model represents a research limitation, the chicken model was thus developed to investigate the long-term effects in this study. Although not every LAB strain showed the same antagonistic ability against *C. jejuni* in both nematodes and chickens, the significant correlation between the ability of LAB in clearing *C. jejuni* in chicken and that in
elevating the survival rate of *C. jejuni*-infected nematodes still indicated that *C. elegans* is a good model organism for screening for *C. jejuni*-resistive LAB strains on a large scale.

**Conclusion**

This study established a *C. elegans* life-span assay capable of measuring the response of worms to *C. jejuni*. Different LAB had different effects on the response of *C. elegans* to infection with *C. jejuni*. The inhibition of *C. jejuni* intestinal colonisation may have been one of the mechanisms through which LAB protected *C. elegans*, and the protection offered by LAB may also have derived partly from their activation of the nematode’s defence immune genes. This *C. elegans* life-span model can be used to screen for *C. jejuni*-antagonistic LAB on a large scale.

**Methods**

*C. elegans*, LAB and *C. jejuni*

*C. elegans* N2 Bristol wild-type strain (Caenorhabditis Genetics Center, Minneapolis, University of Minnesota) was used in the study. The *C. elegans* was maintained and cultivated at 20°C. S medium, M9 buffer and nematode growth medium (NGM) were used to cultivate the nematode and to conduct life-span experimental studies. The procedures for the cultivation, maintenance and synchronization of the nematode have been reported[48]. *E. coli* OP50 was grown at 37°C for 24 h in Luria-Bertani medium to a bacterial concentration of $10^8$ colony-forming units (CFU) per mL. It was used as food for *C. elegans*.

Six LAB were purchased from the American Type Culture Collection (ATCC) or the Japan Collection of Microorganisms and 38 were isolated from human faeces from different habitats and traditional fermented food. Samples of healthy human faeces and traditional fermented foods were collected and the LAB were enriched in sorbitol GM 17 medium at 35°C for 12 h. After gradient dilution, the enriched samples were coated on GM 17 medium plate with 0.02% cresol violet and cultured for 24h. Some single bacterial colonies in line with LAB morphology were selected, and their Gram property determined by Gram staining. The selected LAB were identified through 16S gene sequencing. All the isolates were deposited at the Culture Collection of Food Microorganisms at Jiangnan University (Table 1 and S1). In this study, no human experiments was conducted. The human volunteers were not expected to encounter risk or discomfort in the process of faecal sampling and written informed consent to handle faecal samples for public health purposes was obtained from the volunteers or, where relevant, their legal guardians. The whole genome sequence of 12 isolates, namely N8, N9, 422, 427, 430, Z5, L103, X13, JS-SZ-1-5, JS-WX-9-1, 9-5 and H27-1L were aligned and identified as new strains of the corresponding species reported. The other 26 isolates are assumed tentative strains, for they were isolated from samples of different origin, geographical location and span over seven years. All of the LAB were cultivated in deMan, Rogosa and Sharpe (MRS) agar at a 2% (v/v) inoculum size at 37°C for 18 to 20 h. The isolates were kept at -80°C in 30% glycerol for long-term storage. The LAB were sub-cultured twice before being used in experiments at 2% (v/v) inoculum size.

The *C. jejuni* strain NCTC 11168 was purchased from the ATCC. A *C. jejuni* selective supplement (Oxoid) and 5% sterile sheep blood were added to Columbia blood agar base plates (Oxoid, UK). *C. jejuni* strains were grown in this medium under special gas conditions (5% O₂, 10% CO₂, 85% N₂) for 48 h at 37°C.
Life-span experimental analysis of *C. elegans*

The nematodes were synchronised as previously described[27]. After synchronisation, the eggs were placed on *E. coli* OP50 NGM plates for 72 h until they reached the L4 stage. Most of the experiments lasted 25 days. All experiments were carried out at 20°C, and at least three independent replications were performed for each assay.

To build a model for the experimental analysis of worm death induced by *C. jejuni*, 10⁹ CFU/mL of *C. jejuni* was prepared from the L4 stage worms at 25 days incubation. Each group had 80-100 worms. As shown in Fig. 1A, worms fed only with *E. coli* OP50 formed the negative control group (E/day0 group). In an experiment undertaken to evaluate the role of LAB in protecting the worms against death caused by *C. jejuni*, the worms were either fed 10⁸ CFU/mL *E. coli* OP50 or 10⁹ CFU/mL LAB strains for the first 3 days. After 72 h of incubation, the worms were moved to new 6 cm plates with *C. jejuni* at a concentration of 10⁹ CFU/mL. The worms that were first fed *E. coli* OP50 (72 h) and next *C. jejuni* formed the *C. jejuni* reference group (E/day0+C/day3 group); those treated with LAB (72 h) before *C. jejuni* were regarded as the LAB protection groups (L/day0+C/day3 groups). A worm was considered dead when it failed to respond to gentle touch with a worm picker. The numbers of live worms were recorded, and the probability of their survival was calculated as described previously[27].

**Examination of bacterial load in the intestine of *C. elegans***

The numbers of *E. coli* OP50, *Lactobacillus* and *C. jejuni* in the nematodes’ intestine were determined with some modification of the method described previously [27]. Worms were incubated with *E. coli* OP50, a *Lactobacillus*, or *C. jejuni*, and sampling (50 worms per sample) was done every 2 days. After surface sterilization, the worms were mashed mechanically with a pellet pestle motor, re-suspended in the M9 buffer, and inoculated onto eosin-methylene blue medium (EMB medium), MRS or Columbia blood agar for counting of *E. coli* OP50, *Lactobacillus* and *C. jejuni*, respectively. At least three independent replications were performed for each assay.

**Measurement of body size and pharynx pumping**

The live worms were examined for their body size measurements every 2 days and their pharynx pumping on the third day until they were infected with *C. jejuni*. Images of adult nematodes were taken with a VCT-VBIT digital microscope (Shimadzu, Kyoto, Japan) and analyzed using the ImageJ software. In this system, the area of the worm’s projection was estimated automatically and used as an index of body size. And the worm’s pharynx pumping was measured per 30s.

**Evaluation of the effects on the growth of *C. jejuni* by co-culturing *E. coli* OP50**

The growth of *C. jejuni* co-cultured with *E. coli* OP50 was determined by the following method as previously described. The *C. jejuni* cells (10⁷ CFU/mL) suspended in antibiotic-free brain heart infusion broth (BHIB) containing 5% serum were incubated under microaerophilic conditions for 48 h at 37°C in the presence of a 10% volume of live *E. coli* OP50 (10⁷ CFU/mL). The viability of *C. jejuni* was evaluated from the number of
viable CFUs in *C. jejuni* culture as described above on *C. jejuni*-selective plates. At least three independent replications were performed for this assay.

**RNA extraction, reverse transcription and quantitative real-time PCR analysis**

The whole RNA of *C. elegans* and of the bacteria used in the life-span experimental was extracted. About 100 worms were prepared for the lysates. The RNA was extracted as previously described[27].

The transcription of mitogen-activated protein kinase (MAPK) pathway genes (*tir-1*, *nsy-1*, *sek-1* and *pmk-1*), antimicrobial peptide genes (*spp-1*, *clec-85*, *abf-2*, *clec-60* and *lys-7*), Daf-16 pathway genes (*daf-16* and *age-1*), a TGF-β pathway gene (*dbl-1*) and antioxidant genes (*skn-1* and *bar-1*) in *C. elegans* was determined by quantitative polymerase chain reaction (qPCR)[49]. *GapA*, as a housekeeping gene, was used to determine the levels of mRNA transcription of the *C. elegans* immune genes and to normalize the input amounts of RNA. PCR primers specific to each of the genes were experimentally validated and used in the RT-qPCR assay. The annealing temperature of the RT-qPCR assay was 56°C. The delta Ct method was used to analyse the RT-qPCR data and to determine the relative abundance of the target genes (fold changes) (fold changes)[50, 51].

**Induction of acute ileitis for *C. jejuni* infection and LAB intervention in mice**

Three-week-old female C57BL/6 mice obtained from Shanghai Laboratory Animal Center (Shanghai, China) were used in the experiments. Eight mice were randomly housed in each cage, with a 12-h light-dark cycle and a controllable environment (humidity, 45% ± 5%; temperature, 22°C ± 2°C). All the experimental procedures (#JIPD2017030) had been approved by the Animal Care and Use Committee at the Jiangsu Institute of Parasitic Diseases. All the experiments conformed to the Ministry of Science and Technology of China's Guide for the Care and Use of Laboratory Animals.

To induce ileitis, the C57BL/6 mice were infected orally with 100 *T. gondii* cysts (ME49 strain; obtained from the Jiangsu Institute of Parasitic Diseases, Remington, Wuxi, China) in 0.3 mL phosphate-buffered saline (PBS, pH 7.4) by gavage, as described previously [52]. As shown in Fig. 1B, for the 4 days thereafter, the mice were successively (at 1 h intervals) treated with LAB (10^9 CFU in 0.3 mL or 0.3 mL PBS) and *C. jejuni* NCTC 11168 (10^9 CFU in 0.3 mL) by gavage on 2 consecutive days. The *C. jejuni* loads in their faeces were checked 3 days later. The faeces were resuspended in sterile PBS and serially diluted. The diluted samples were spread on Columbia blood agar with a *C. jejuni* selective supplement and incubated at 5% oxygen concentration at 37°C for 48 h. After incubation, the numbers of *C. jejuni* in the samples were counted. The mice were euthanized with CO₂ after experiment. For each mice, the treatment based on the different LAB, the blood collection and execution and analysis of indicators were all done by different investigator. The first investigator was the only person aware of the treatment group allocation.

**C. jejuni** infection and LAB intervention in chicken

White leghorn chicken eggs (Jinan Baizhun Biologic Inspection Company, Ltd., China) were maintained in an egg incubator until the chicks hatched. About 24 hours after hatching, 8 chicks were randomly assigned to several groups. All the experimental procedures were approved by the Animal Care and Use Committee at Jiangsu Nannong Hi-technology company, LTD. All the experiments adhered to the Ministry of Science and Technology of China's Guide for the Care and Use of Laboratory Animals.
As shown in Fig. 1C, bacterial cells were washed and resuspended in ice-cold PBS prior to inoculation. All birds were administered $10^8$ CFU of *C. jejuni* NCTC 11168 in a 0.3 mL suspension by oral gavage. Twenty-four hours after oral gavage, LAB ($10^8$ CFU in 0.3 mL) were orally administered daily to 11 groups (in total 88 birds) of *C. jejuni*-inoculated birds for two weeks. PBS was administered to the remaining one *C. jejuni* group of birds (8 birds). Chicks were euthanized with CO$_2$ at 23 days post-inoculation, and the cecal contents were diluted in ice-cold PBS to 0.1 g/mL. Ten-fold serial dilutions of each sample were prepared and then plated on Columbia blood agar with a *C. jejuni* selective supplement and incubated at a 5% oxygen concentration at 37°C for 48 h. For each mice, the treatment based on the different LAB, the blood collection and execution and analysis of indicators were all done by different investigator. The first investigator was the only person aware of the treatment group allocation.

**Statistical analysis**

GraphPad Prism 5.0 and Origin 9.0 were used to perform the statistical analysis, and SPSS Statistics 20.0 was used for the significance analysis. The data were expressed as means ± standard deviations (SDs). Kaplan-Meier survival analysis was used to assess the survival rate of *C. elegans*. Correlation analysis of the *C. elegans* groups was conducted using SPSS Statistics 20.0 and Origin 9.0. The groups were compared using a two-tailed Student's t-test, and a two-sided p value of less than 0.05 was considered statistical significance. Mean values with different superscript letters over the bars are significantly different ($p < 0.05$).

**Abbreviations**

*C. jejuni*. *Campylobacter jejuni*; LAB: lactic acid bacteria; *E. coli*. *Escherichia coli*; *C. elegans*. *Caenorhabditis elegans*; *T. gondii*. *Toxoplasma gondii*

**Declarations**

**Ethics approval and consent to participate**

All the mice experimental procedures (#JIPD2017030) had been approved by the Animal Care and Use Committee at the Jiangsu Institute of Parasitic Diseases. All the chicken experimental procedures were approved by the Animal Care and Use Committee at Jiangsu Nannong Hi-technology company, LTD. All the experiments adhered to the Ministry of Science and Technology of China's Guide for the Care and Use of Laboratory Animals.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Competing interests**
The authors declare that they have no competing interests.

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Authors' contributions

GW and XJ conceived and designed the experiments; XJ, YH and XC performed the experiments; XJ and GW analyzed the data; YZ, YL, JZ, HZ and WC contributed reagents/materials/analysis tools; XJ and GW wrote the paper. All authors contributed to manuscript revision, read and approved the submitted version.

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### Tables

**Table 1**

Statistical analysis of the protection effects of LAB stains on *C. elegans* infected by *C. jejuni* NCTC 11168

| Groups<sup>b</sup> | Species<sup>a</sup> | Year of isolation | Location of isolation | Origin | Survival (%) | DT50<sup>c</sup> (day) | P    |
|--------------------|--------------------|--------------------|-----------------------|--------|--------------|-------------------|------|
| E/day0+C/day3      | –                  | –                  | –                     | –      | 20.16        | 6.83              |      |
| E/day0            | –                  | –                  | –                     | –      | 55.78        | 14.90             | 0.01 |
| 13-7              | *L. salivarius*    | 2016               | Bama, Guangxi         | Healthy adult feces | 47.09        | 14.01             | <0.01|
| Z5                | *L. salivarius*    | 2013               | Huhehaote, Neimengu   | Traditional koumiss | 40.36        | 12.64             | <0.01|
| N9                | *L. plantarum*     | 2010               | Leshan, Sichuan       | Traditional koumiss | 43.28        | 13.70             | <0.01|
| 422               | *L. fermentum*     | 2010               | Leshan, Sichuan       | Traditional pickles | 15.09        | 6.06              | 0.92 |
| B                 | *L. fermentum*     | 2010               | Wuxi, Jiangsu         | Healthy infant feces | 18.02        | 7.38              | 0.88 |
| G14               | *L. reuteri*       | 2016               | Bama, Guangxi         | Healthy adult feces | 20.01        | 7.31              | 0.87 |

<sup>a</sup>Summary of two or more separate experiments. Survival of worms on the last day (day 13) of the assays was estimated with the Kaplan-Meier survival analysis.

<sup>b</sup>E/day0+C/day3: treatment with *E. coli* OP50 in the first 3 days and then with *C. jejuni* when *C. elegans* at L4 stage. E/day0: treatment with *E. coli* OP50 during the whole experiment when *C. elegans* at L4 stage. LAB: treatment with LAB in the first 3 days and then with *C. jejuni* when *C. elegans* at L4 stage. The time of L4 stage nematodes before fed any thallus was considered 0 day.

<sup>c</sup>DT50, the time at which half of the worms were dead.
Table 2

Correlation analysis of LAB colonization and *C. jejuni* load in the intestine and survival of *C. elegans*

| Comparison Groups | Pearson Correlation |
|-------------------|---------------------|
| Colonization of LAB | *C. jejuni* load | R = -0.424* |
| Colonization of LAB | Survival of *C. elegans* | R = 0.517** |
| *C. jejuni* load | Survival of *C. elegans* | R = -0.615** |

* Indicates statistically significant differences at \( p < 0.05 \).

** Indicates statistically significant differences at \( p < 0.01 \).

**Figures**
Figure 1

Flow chart of LAB interventions in C. elegans, mice and chicken. (A) C. elegans experimental design during L4 stage. (B) Experimental design for mice. (C) Experimental design for chicken.
Figure 2

Establishment of a life-span, body size and C. jejuni load in C. elegans assay. (A) Flow chart for each experimental group. (B) Life-span of C. elegans treated with E. coli and C. jejuni in different ways. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. (C) Body size of C. elegans treated with E. coli and C. jejuni in different ways. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. (D) C. jejuni load in C. elegans treated with E. coli and C. jejuni in different ways. Day 0 marked the point at which the nematodes were first fed C. jejuni.
Figure 3

Differential effects of LAB on the survival of C. elegans infected with C. jejuni. E/day0+C/day3: treated with E. coli OP50 for the first 3 days and then with C. jejuni during the L4 stage. LAB: treated with LAB for the first 3 days and then with C. jejuni during the L4 stage. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus.

Figure 4

LAB load in the intestine of C. elegans. (A) LAB: treated with LAB for the first 3 days and then with C. jejuni during the L4 stage. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. (B) LAB load in the intestine of C. elegans infected with C. jejuni on day 6. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. The graphs show means ± SDs. Column labelled with different superscript letters (a, b) showed significant differences (p<0.05). Any two columns with same superscript letter.
Figure 5

C. jejuni load in the intestine of C. elegans. (A) E/day0+C/day3: treated with E. coli OP50 for the first 3 days and then with C. jejuni during the L4 stage. LAB: treated with LAB for the first 3 days and then with C. jejuni during the L4 stage. Day 0 marked the point at which the nematodes were first fed C. jejuni. (B) The C. jejuni load in the intestine of C. elegans infected with C. jejuni on day 6. Day 0 marked the point at which the nematodes were first fed C. jejuni. The graphs show means ± SDs. Column labelled with different superscript letters (a, b) showed significant differences (p<0.05). Any two columns with same superscript letter.

Figure 6

Effects of LAB on body size and pharynx pumping of C. elegans. (A) E/day0+C/day3: treated with E. coli OP50 for the first 3 days and then with C. jejuni during the L4 stage. LAB: treated with LAB for the first 3 days and then with C. jejuni during the L4 stage. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. (B) Body size (area) of C. elegans treated with LAB and C. jejuni on day 8. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. (C) Effects of LAB on the pharynx pumping of C. elegans infected by C. jejuni. Pharynx pumping (per 30s) of C. elegans treated with LAB and C. jejuni on day 8. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. The graphs show means ±
SDs. Column labelled with different superscript letters (a, b) showed significant differences (p<0.05). Any two columns with same superscript letter.

Figure 7

Differential effects of LAB on transcription of immune genes of C. elegans on day 6. E/day0+C/day3: treated with E. coli OP50 for the first 3 days and then with C. jejuni during the L4 stage. LAB: treated with LAB for the first 3 days and then with C. jejuni during the L4 stage. Day 0 marked the arrival of the nematodes at the L4 stage before being fed thallus. * Indicates statistically significant differences at p < 0.05.

Figure 8
Differential effects of LAB on C. jejuni load in mice infected by C. jejuni. (A) Culturable C. jejuni in mice faeces on day 5. The graphs show means ± SDs. Column labelled with different superscript letters (a, b, c) showed significant differences (p<0.05). Any two columns with same superscript letter. (B) Correlation analysis of ability of LAB strains to clear C. jejuni in infected mice on day 5 and the survival rate of C. jejuni-infected nematodes under LAB treatment.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 9**

Differential effects of LAB on C. jejuni load in chicken infected by C. jejuni. (A) Culturable C. jejuni in chicks’ cecal contents on day 23. The graphs show means ± SDs. Column labelled with different superscript letters (a, b, c) showed significant differences (p<0.05). Any two columns with same superscript letter. (B) Correlation analysis of ability of LAB strains to clear C. jejuni in infected chicks’ cecal contents and the survival rate of C. jejuni-infected nematodes under LAB treatment.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEchecklist.docx
- SupplementaryMaterial.docx