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Use of *Caenorhabditis elegans* for Preselecting *Lactobacillus* Isolates To Control *Salmonella* Typhimurium

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ABSTRACT

Host-specific probiotics have been used to control enteric pathogens, including foodborne pathogens, in food animal production. However, evaluation of the efficacy of these probiotics requires costly in vivo assays in the target animal. The nematode *Caenorhabditis elegans* has been used for prescreening of antimicrobial agents and for studies of host-pathogen interactions. In the present study, 17 *Lactobacillus* isolates from chicken and pig intestines were tested with *C. elegans,* and the ability of these isolates to prevent death from *Salmonella* infection was variable. Two *Lactobacillus* isolates (S64, which gave full protection, and CL11, which gave no protection) were further studied. Both isolates exhibited a similar colonization profile in the *C. elegans* intestine. Although different culture fractions of CL11 were not protective, both live and heat-killed S64 cells provided full or partial protection of *C. elegans* from death caused by *Salmonella* infection. In contrast, different culture fractions from both isolates had similar effects on the colonization of the nematode intestine by *Salmonella* Typhimurium DT104. Our preliminary results from a pig performance trial revealed a correlation between the degree of protection in the *C. elegans* survival assay and the performance of 35-day-old weaned piglets that were treated with the same *Lactobacillus* isolates, suggesting that *C. elegans* can be used as a laboratory animal model for preselecting probiotics for control of *Salmonella* infections.

Human salmonellosis has been a significant food safety issue for decades and often is associated with the consumption of poultry and pork products *(5, 7, 8, 26, 30, 33).* To reduce the risk of salmonellosis, efforts from every step in food production (including food animal production), processing, and services are required to eliminate *Salmonella* contamination. Control of *Salmonella* infection in poultry and swine production is an important and critical step in reducing the contamination. Probiotic bacteria including lactobacilli have been suggested as an alternative control method for reducing infection by enteric pathogens *(25).* Probiotic bacteria are abundant in the intestine of animals *(16, 17, 23)* and provide a variety of benefits to their hosts *(9, 32).* To identify effective probiotics for food animal production, selection through in vitro and in vivo studies usually is required. However, selection based on in vivo studies is limited by high cost and the low efficiency of using food animals to identify the beneficial and safe species and/or strains. The nematode *Caenorhabditis elegans* has been used as a laboratory animal model for prescreening antimicrobial agents *(31)* because these worms can be infected and killed by a remarkable number of human pathogens, including *Salmonella enterica* and *Staphylococcus aureus (2, 39). C. elegans* has a short reproductive life cycle, is easy to culture, and is suitable for genetic analysis *(37).* In previous studies, a broad overlap has been found between the bacterial virulence factors required for pathogenesis in mammals and for death in *C. elegans (12, 41).* Thus, simple feeding-based pathogenicity assays with survival after infection as the biomarker of morbidity and mortality from diseases facilitate high-throughput prescreening of antimicrobials. Ikeda et al. *(18)* evaluated the influence of lactic acid bacteria on the longevity of *C. elegans* infected with *S. enterica* serovar Enteritidis. These researchers found that all isolates produced effective protection against *Salmonella*-induced death. In the present study, we performed a preliminary investigation of the possible correlation of the probiotic effects of lactobacilli in nematodes and pigs. The specific objectives were (i) to assess the suitability of *C. elegans* as an in vivo prescreening model for selection of *Lactobacillus* isolates from chicken or pig intestines, (ii) to select and analyze the probiotic candidates for their suitability for controlling *Salmonella* Typhimurium infection, and (iii) to perform a preliminary nematode-pig correlation analysis of probiotic effects.

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TABLE 1. *Statistical analysis of the protection effect of* Lactobacillus *isolates on* C. elegans *infected with* Salmonella[a]

Isolate[b]	Origin[c]	Putative identity[d]	Survival (%)	95% CI (%)	*P* value[e]	
					L+S. vs *E. coli*	L+S vs E+S
E+S			17.2	5.79	<0.0001	
E. coli OP50			71.4	12.68		<0.0001
ALB2	C	*Lactobacillus salivarius*	39.4	11.98	<0.0001	<0.0001
ALB6	C	*L. salivarius*	54.7	12.43	0.0180	<0.0001
ALB7	C	*L. salivarius*	26.6	10.82	<0.0001	0.0289
ALB10	C	*L. salivarius*	24.4	11.59	<0.0001	0.0255
ALB11	C	*L. crispatus*	18.3	11.46	<0.0001	0.4038
CL9	P	*L. reuteri*	74.6	12.42	0.8940	<0.0001
CL10	P	*L. acidophilus*	4.9	4.93	<0.0001	0.1360
CL11	P	*L. casei*	9.9	7.51	<0.0001	0.8820
CL12	P	*L. amylovorus*	15.5	9.32	<0.0001	0.3779
L2	C	*L. rhamnosus*	58.6	11.96	0.0537	<0.0001
L3	C	*L. rhamnosus*	71.3	11.22	0.6969	<0.0001
LB1	C	*L. zeae*	66.3	10.98	0.3565	<0.0001
LB2	C	*L. rhamnosus*	32.5	10.46	<0.0001	0.0053
LB4	C	*L. rhamnosus*	78.8	9.87	0.5700	<0.0001
S64	P	*L. reuteri*	75.6	10.41	0.6369	<0.0001
SG1	C	*L. salivarius*	14.1	11.36	<0.0001	0.5060

[a] Summary of two or more separate experiments. Survival of worms on the last day (day 9) of the assays with 95% confidence interval (CI) was estimated with the Kaplan-Maier survival analysis.
[b] E+S, treatment with *E. coli* OP50 and then *Salmonella* Typhimurium DT104.
[c] C, isolates from chickens; P, isolates from pigs.
[d] Putative species identity was determined by BLAST analysis of sequences of 16S rRNA genes. Sequence similarities between the isolates and the 16S rDNA sequence database were 98 to 100% except for isolate SG1 (93%).
[e] Comparisons of survival curves. L+S, treatment with *Lactobacillus* and then *Salmonella* Typhimurium DT104.

MATERIALS AND METHODS

Nematodes, bacteria, and media. A *C. elegans* temperature-sensitive mutant strain (SS104) was used in the present study (Genetics Center, University of Minnesota, Minneapolis). The strain is unable to propagate at 25°C or higher and was therefore cultured and maintained at 15°C and incubated at 25°C for life span assays. Standard procedures described by Stiernagle *(40)* were followed for all general worm maintenance and assays. *Escherichia coli* OP50 (10^8 CFU/ml) grown in Luria-Bertani broth or agar at 37°C for 12 h was used as a diet to feed the nematode *(11)*. Nematode growth medium (NGM) and S medium *(40)* were used for culturing and *C. elegans* life span assays.

Salmonella Typhimurium DT104 strain SA970934 is a porcine multiantibiotic-resistant isolate *(34, 35)*. This strain was cultured on tryptic soy broth (TSB) or tryptic soy agar at 37°C for 16 h. Brilliant green sulfa (BGS) agar was used to examine *Salmonella* colonization in the intestine of *C. elegans*. Lactobacilli were isolated from adult pig or chicken intestine (mainly from the cecum). Their putative identity was determined by comparison of partial sequences of 16S rRNA genes corresponding to *E. coli* 16S rRNA bases 400 to 1,050 in the GenBank, EMBI and DBJI nonredundant nucleotide databases using BLASTn *(15)* (Table 1). Either de Man Rogosa Sharpe (MRS) broth or agar was used to culture *Lactobacillus* isolates at 37°C for 18 to 24 h in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2.

Antimicrobial activity assay. Antimicrobial activity of 17 *Lactobacillus* isolates against *Salmonella* Typhimurium DT104 was estimated by the ''spot-on-the-lawn'' assay as described by Lewus and Montville *(22)*. Antagonism was detected by the formation of a growth inhibition halo of *Salmonella* around individual *Lactobacillus* inoculation sites. The size of the inhibition halo was recorded for comparison of antimicrobial activity.

Assay for the life span of *C. elegans*. The longevity of *C. elegans* after various treatments was determined using the method described by Ikeda et al. *(18)*. The worms were hatched from eggs that had been released from adult worms and treated with sterile water containing 0.5 M NaOH and a 0.5% solution of freshly prepared sodium hypochlorite (bleach) for synchronization, as described by Stiernagle *(40)*. After 10 min with vortexing at 2-min intervals, worms were washed twice in S medium via centrifugation (1,300 × *g* for 1 min) and suspension. To prepare nematodes for the assay, 0.5 ml of synchronized eggs (approximately 200 to 300 eggs) were hatched in M9 buffer at 20°C overnight. The L1 larvae were subsequently transferred to NGM agar with a 12-h lawn of *E. coli* OP50 on the surface and incubated at 25°C for 48 to 60 h to reach the L4 stage. After releasing the worms from NGM agar with M9 buffer and three washes in S medium via centrifugation (1,300 × *g* for 2 min) and suspension, 10 to 15 worms were placed in each well of a 24-well titer plate containing 2 ml of S medium and were incubated at 25°C during the assay. Each treatment was repeated three times with a total of 30 to 45 worms per experiment. The assays generally lasted for 8 to 9 days unless indicated otherwise. The S medium was not changed during the assays because incubation of *C. elegans* in the medium at 25°C in the presence of *E. coli* OP50 or one of the tested *Lactobacillus* isolates had no influence on the life span of the nematode within the duration of the assays. The start day when worms were fed *E.*

coli OP50 (10^8 CFU/ml) or a *Lactobacillus* isolate (10^9 CFU/ml for establishing the assay and 10^8 CFU/ml for the assays to examine the effect of *Lactobacillus* isolates) was designated as day 0. After 18 h of incubation (designated as day 1), the worms were infected with *Salmonella* Typhimurium DT104 (10^9 CFU/ml). Worms treated with only *E. coli* OP50 served as the negative control, and worms treated with *E. coli* OP50 (18 h) and then with *Salmonella* Typhimurium DT104 served as the positive control. To evaluate the effect of a *Lactobacillus* isolate for protecting the nematode from *Salmonella*-induced death, the same procedure as used for the positive control was followed except a particular *Lactobacillus* isolate was used instead of the *E. coli* OP50, unless otherwise indicated. The *E. coli* OP50, *Salmonella* Typhimurium DT104, and *Lactobacillus* used for the *C. elegans* life span assays were all from cultures in the early stationary phase. The bacteria were washed twice in S medium by centrifugation and suspension before being fed to the nematodes. To test the protective effect of different culture fractions of *Lactobacillus,* live or heat-killed *Lactobacillus* cells (killed by exposure to 70°C for 20 min) were used instead of *E. coli* OP50 in the assays. When the extracellular culture fluid (ECF) from *Lactobacillus* cultures was tested, *E. coli* OP50 was included in the assay mixtures as the nematode food. The ECF was prepared by separating culture supernatant from the cells via centrifugation at 10,400 × *g* for 10 min at 4°C *(14).* The heat-treated cells were examined by microscopy and evaluated in growth tests. The *Lactobacillus* cells remained intact but failed to grow (data not shown). To determine the life span or survival of *C. elegans,* the number of live worms was recorded daily, and the percentage of surviving worms was calculated by the following formula: survival (%) = (live worms/total worms used) × 100. A worm was considered dead when it failed to respond to touch.

Examination of bacterial (*Lactobacillus* and *Salmonella*) colonization of the *C. elegans* intestine. Examination of *Salmonella* and *Lactobacillus* colonization of the intestine of *C. elegans* was conducted using a previously described method *(12)* with some modifications. Ten L4 stage worms (per sample) were incubated with *Salmonella* Typhimurium DT104 or each *Lactobacillus* isolate, and sample worms were collected every 2 days. These worms were washed twice in M9 buffer by centrifugation (1,300 × *g* for 1 min) and suspension and then treated with 100 μl of 70% ethanol for 20 s for surface sterilization. After the ethanol, worms were immediately immersed in 1 ml of M9 buffer to reduce the final concentration of ethanol to approximately 6.4%. The worms were then washed two more times with M9 buffer. These surface-sterilized worms were subsequently examined; they had intact bodies, and no growth of bacteria was apparent on the nutrient agar plates, indicating that no live bacterial cells were associated with the surface of the worms (data not shown). After surface sterilization, the worms were mashed mechanically with a pellet pestle motor, resuspended in M9 buffer, and plated on BGS or MRS agar for enumeration of *Salmonella* and *Lactobacillus,* respectively.

Pig trial, sample collection, and analyses. A growth performance trial with weaned pigs was carried out at a pig farm with a history of infectious diarrhea. This farm in Beijing, China, was selected by the Feed Research Institute (Chinese Agricultural Academy of Sciences, Beijing, China) to examine possible effects of our *C. elegans* preevaluated *Lactobacillus* isolates on pig performance. The protocol for the animal experiment was approved by the local animal care and use committee of the institute, and the animals were handled in a humane manner in accordance with the guidelines established by the Danish Ethical Commission.

TABLE 2. *Ingredients and nutritional composition of diet for weaned pigs*[a]

Composition	Amount
Ingredient	
Maize	66.00%
Soybean meal	19.00%
Bran	6.00%
Fish meal	5.00%
Calculated nutrition	
Digestible energy	13.40 MJ/kg
Crude protein	18.20%
Lysine	1.21%
Methionine	0.32%
Cystine	0.29%
Threonine	0.68%
Tryptophan	0.21%
Total calcium	0.80%
Total phosphorus	0.65%
Available phosphorus	0.40%

[a] Diet was a typical maize-soybean meal basal formula without premix for weaned pigs. The premix formula in this diet contained vitamin A (6 mg), vitamin D_3 (8 mg), vitamin E (30 mg), vitamin K (3 mg), vitamin B_2 (27 mg), vitamin B_6 (2 mg), vitamin B_{12} (30 μg), biotin (80 μg), folic acid (8 mg), nicotinic acid (24 mg), Na (NaCl, 3 g), Zn ($ZnSO_4$, 165 mg), Fe ($FeSO_4$, 165 mg), Mn ($MnSO_4$, 33 mg), Cu ($CuSO_4$, 165 mg), I (CaI_2, 297 μg), Se (Na_2SeO_3, 297 μg). All ingredients listed were supplied per kilogram of diet.

Ninety-six weaned piglets (Duroc × Landrace × Large Yorkshire) that were 35 days of age and similar weight were allocated to eight dietary treatments (three pens per treatment with four pigs per pen) on the basis of weight and gender in a randomized complete block design. Treatment 1 was the basal diet (Table 2) in solid form (control I), treatment 2 was the basal diet in liquid form (solid feed:water = 1:1.6) (control II), and treatments 3 through 8 were the basal diet fermented with one of the following *Lactobacillus* isolates: CL11, L3, LB2, LB1, ALB2, and S64. The fermented feed was prepared by incubating the basal diet in liquid form that had been inoculated with a *Lactobacillus* isolate (3×10^6 cells per ml). The fermentation was conducted at room temperature (approximately 20°C) for 48 h or more in a closed tank until the pH of the feed was reduced to 4. All the pigs were housed between 17 and 23°C with free access to water and feed during the trial. The first week of the pig trial was considered to be a period for each group of pigs to adapt to the tested diet. Pig performance was then monitored during the following 4 weeks. The day when recording of pig performance began was designated as the beginning of the experiment (day 0). Pigs were weighed in the early morning on day 0 and weekly thereafter to determine growth performance. Unconsumed feed was recorded daily during the trial to calculate average daily feed intake based on dry weight. Average daily body weight gain (ADG) and feeding efficiency represented by the ratio of feed intake to body weight gain (F/G) were used to measure pig growth performance. Diarrhea of the weaned pigs was recorded daily by the same person (who was blind to treatments) from day 0 to the end of the trial. The diarrheal index was used as a measure of diarrhea severity according to the standard described by Miller et al. *(28).*

Statistical analysis. Statistical computations were performed using the GraphPad Prism software (version 4, San Diego, CA)

and the Statistical Analysis System (SAS release 9.1, SAS Institute Inc., Cary, NC). Survival curves for *C. elegans* were compared with the Kaplan-Maier survival analysis followed by a log-rank test. Duncan's multiple comparison test was used to determine differences among treatment means for pig performance. $P < 0.05$ was taken to indicate statistical significance. Correlation analysis was used to determine the relationship between the degree of protection in the *C. elegans* survival assay, as indicated by the Kaplan-Maier survival analysis, and the performance of pigs treated with the same isolates.

RESULTS

Growth inhibition of *Salmonella* Typhimurium DT104 by *Lactobacillus* isolates. The spot-on-the-lawn assay revealed that all 17 tested *Lactobacillus* isolates inhibited growth of *Salmonella* Typhimurium DT104. The growth inhibition halos ranged from 19 to 25 mm in diameter, with the majority (12 isolates) 21 to 23 mm. The ECFs recovered from tested *Lactobacillus* cultures all had a pH of about 4.2 and a similar degree of inhibition of *Salmonella* Typhimurium DT104, as indicated by little growth of the pathogen in the TSB. However, neutralized ECFs (pH 7.0) produced little inhibition of the pathogen. The optical density at 600 nm (OD_{600}) of the *Salmonella* cultures in the presence of the neutralized ECF ranged from 1.37 to 1.82 after 18 h of incubation, whereas the culture without the ECF had an OD_{600} value of 1.86.

Establishing *C. elegans* assay to estimate the effect of *Salmonella* infection and *Lactobacillus* protection. When *C. elegans* was fed *E. coli* OP50 only, minimal death of the nematode was observed after 12 days of incubation (Fig. 1A). A similar observation was obtained when the nematode was fed *Lactobacillus* isolate S64 only at both tested doses (10^8 and 10^9 CFU/ml). Isolate S64 was one of the isolates that produced the highest level of inhibition of *Salmonella* Typhimurium DT104 in the spot-on-the-lawn assay. In contrast to the effects of *E. coli* OP50 and *Lactobacillus* isolate S64, treatment with *Salmonella* Typhimurium DT104 caused the death of all the worms within 4 to 5 days. Ingestion of *Lactobacillus* isolate S64 offered effective protection to the nematode from *Salmonella*-induced death within 9 days, regardless of whether the *Lactobacillus* isolate was ingested before or after the introduction of the *Salmonella* (Fig. 1B). The longevity of *Lactobacillus*-treated worms was comparable to that of worms fed *E. coli* OP50 only after 8 days of *Salmonella* infection. Therefore, all the remaining assays in this study were carried out for a maximum of 9 days, and all nematodes were treated with *Lactobacillus* before *Salmonella* infection.

Effect of *Lactobacillus* isolates on the longevity of *C. elegans* infected with *Salmonella*. All 17 *Lactobacillus* isolates that inhibited the growth of *Salmonella* Typhimurium DT104 in the spot-on-the-lawn assay were assessed for their protection of *C. elegans* from *Salmonella*-caused death. On the last day (day 9) of the assay, 71 and 17% of the worms from the negative control (fed *E. coli* OP50 only) and positive control (treated with *E. coli* OP50 followed by *Salmonella* Typhimurium DT104), respectively, were still alive (Table 1). The

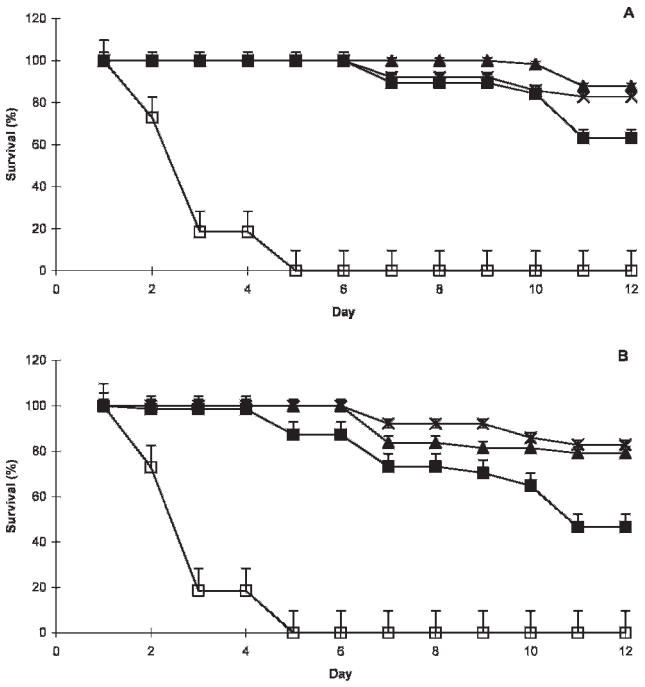

FIGURE 1. *Establishment of* C. elegans *survival assay. (A) Survival of* C. elegans *fed one bacterial species only for 12 days. Worms were fed one of the following:* ×, E. coli *OP50 (normal* C. elegans *food) at 10^8 CFU/ml;* □, Salmonella *Typhimurium DT104 at 10^9 CFU/ml;* ▲, Lactobacillus *isolate S64 at 10^8 CFU/ml;* ■, Lactobacillus *isolate S64 at 10^9 CFU/ml. (B) Effect of pre- and posttreatment with* Lactobacillus *on 12-day survival of* C. elegans *infected with* Salmonella *Typhimurium. Worms were fed one of the following:* ×, E. coli *OP50 only (10^8 CFU/ml);* □, Salmonella *Typhimurium DT104 only (10^9 CFU/ml);* ▲, Salmonella *Typhimurium DT104 (10^9 CFU/ml) for 5 h (followed by three washes in M9 buffer) and then* Lactobacillus *isolate S64 (10^9 CFU/ml) for the remaining days;* ■, Lactobacillus *isolate S64 (10^9 CFU/ml) for 5 h (followed by three washes in M9 buffer) and then* Salmonella *Typhimurium DT104 (10^9 CFU/ml) for the remaining days.*

proportion of live worms ranged from 5 to 79% on the same day in the *Lactobacillus* treatment groups. Four isolates (CL10, CL11, CL12, and SG1) provided no or little protection to the infected worms, which had the level of longevity comparable to that of untreated *Salmonella*-infected worms. The remaining isolates offered various degrees of protection to the nematode from *Salmonella*-induced death. Among these isolates, five (CL9, L3, LB1, LB4, and S64) provided maximum protection to the infected worms, which had a life span similar to that of the worms fed *E. coli* OP50 only. Figure 2 provides a representative case illustrating the effect of *Lactobacillus* during the course of the assay. Although isolate CL11 provided little protection, isolates S64 and CL9 both effectively protected the worms from *Salmonella*-induced death throughout the course of the assay. The worms treated with either the S64 or CL9 isolate had a life span comparable to that of worms fed *E. coli* OP50 only. Treatment with isolate CL11 alone did not reduce the life span of the nematodes compared with treatment with *E. coli* OP50 only (Fig. 3).

Colonization of the intestine of *C. elegans* by *Lactobacillus* isolates. Two *Lactobacillus* isolates, S64

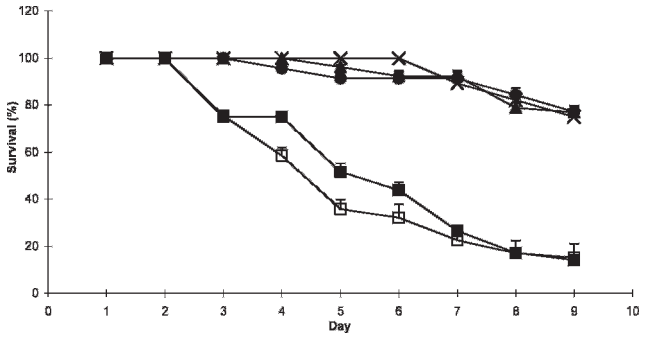

FIGURE 2. *Effect of different* Lactobacillus *isolates on the survival of* C. elegans *infected with* Salmonella *Typhimurium. Control worms were fed* E. coli *OP50 at* 10^8 *CFU/ml for 9 days. For all other treatments, worms were first fed either* E. coli *OP50 or a* Lactobacillus *isolate at* 10^8 *CFU/ml for 18 h and then* Salmonella *Typhimurium DT104 (*10^9 *CFU/ml) for the remaining days. Treatments:* ×, E. coli *OP50 only;* □, E. coli *OP50 and* Salmonella *Typhimurium DT104;* ●, Lactobacillus *isolate S64 and* Salmonella *Typhimurium DT104;* ▲, Lactobacillus *isolate CL9 and* Salmonella *Typhimurium DT104;* ■, Lactobacillus *isolate CL11 and* Salmonella *Typhimurium DT104.*

and CL11, were compared for their ability to colonize the nematode intestine. Although protection from *Salmonella*-induced death differed, with S64 being more protective than CL11 (Fig. 3A and 3C), both isolates had similar and reproducible colonization profiles in the worm intestine (Fig. 3B and 3D). In the assay, neither S64 nor CL11 alone had an impact on the longevity of the worms. The level of colonization by S64 was 10^4 to 10^5 CFU per worm throughout the assay regardless of whether the worms were treated with *Lactobacillus* followed by *Salmonella* or *E. coli* OP50 (Fig. 3B). When the worms were fed *Lactobacillus* only throughout the assay, the level of *Lactobacillus* colonization increased (more than 10^5 CFU per worm) after 4 days of incubation, and the worms appeared to be healthy. Similar observations were obtained with isolate CL11 (Fig. 3D).

Effect of bacterial cells and ECF of *Lactobacillus* isolates on the longevity of *C. elegans* infected with *Salmonella*. To explore possible mechanisms underlying the protection offered by *Lactobacillus,* the effect of different culture fractions of *Lactobacillus* isolate S64, including the ECF and live and heat-killed cells, on the longevity of *C. elegans* infected with *Salmonella* and on the colonization by the pathogen of the nematode intestine were investigated. The live *Lactobacillus* cells offered the highest degree of protection to the nematode from *Salmonella*-induced death (66% of the worms had survived on day 8 of the assay), whereas the ECF provided almost no protection (Fig. 4A). The heat-killed cells provided a moderate level of protection; 51% of the worms had survived on day 8 of the assay. Figure 4B shows the colonization by *Salmonella* Typhimurium DT104 in the intestine of the nematode on different days of the assay after treatment with the *Lactobacillus* ECF or with live or heat-killed *Lactobacillus* cells. On day 2 of the assay, all the worms from different treatment groups had *Salmonella* infections of between approximately 10^5 and 10^6 CFU per

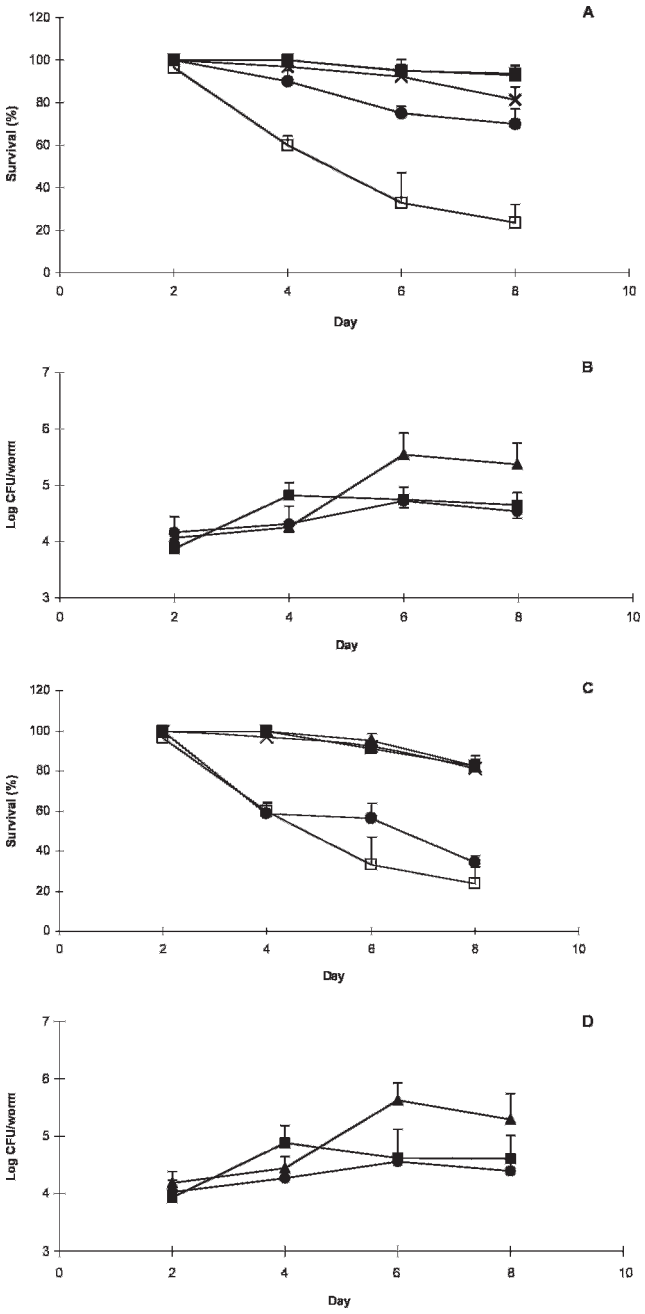

FIGURE 3. *Effect of feeding* Lactobacillus *isolates S64 and CL11 on the survival of* C. elegans *infected with* Salmonella *Typhimurium, and* Lactobacillus *colonization of the worm intestine. (A) Survival rate in the presence of S64. (B) Colonization of the intestine by S64. (C) Survival rate in the presence of CL11. (D) Colonization of the intestine by CL11. Control worms were fed* E. coli *OP50 only or* Lactobacillus *S64 or CL11 at* 10^8 *CFU/ml for 8 days. For other treatments, worms were first fed either* E. coli *OP50 or a* Lactobacillus *isolate at* 10^8 *CFU/ml for 18 h and then either* Salmonella *Typhimurium DT104 or* E. coli *OP50 (*10^9 *CFU/ml) for the remaining days. Treatments:* ×, E. coli *OP50 only;* □, E. coli *OP50 and then* Salmonella *Typhimurium DT104;* ●, Lactobacillus *S64 or CL11 and then* Salmonella *Typhimurium DT104;* ■, Lactobacillus *S64 or CL11 and then* E. coli *OP50;* ▲, Lactobacillus *S64 or CL11 only. The curves of two treatments (*■ *and* ▲*) were overlapped fully in A and partially in C.*

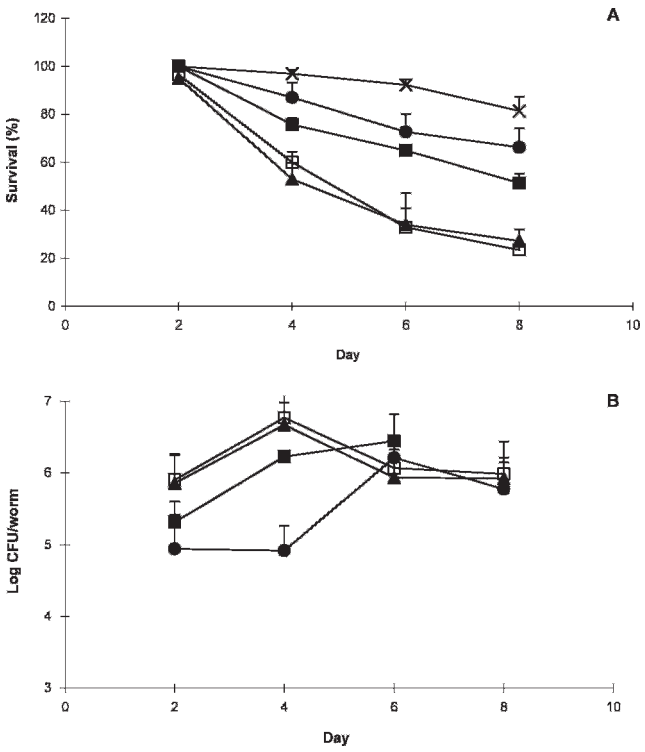

FIGURE 4. *Effect of live and heat-killed* Lactobacillus *cells and the extracellular culture fluid of* Lactobacillus *S64 on the survival of* C. elegans *infected with* Salmonella *Typhimurium and on the colonization of the worm intestine by the pathogen. (A)* C. elegans *survival. (B)* Salmonella *Typhimurium DT104 colonization of the worm intestine. The worms were fed with one of the following for 8 days: ×,* E. coli *OP50 only (10^8 CFU/ml); □,* E. coli *OP50 (10^8 CFU/ml) for 18 h and then* Salmonella *Typhimurium DT104 (10^9 CFU/ml) for the remaining days; ●, live cells of* Lactobacillus *S64 (10^8 CFU/ml) for 18 h and then* Salmonella *Typhimurium DT104 (10^9 CFU/ml) for the remaining days; ■, heat-killed* Lactobacillus *S64 cells (10^8 CFU/ml) for 18 h and then* Salmonella *Typhimurium DT104 (10^9 CFU/ml) for the remaining days; ▲, extracellular culture fluid of* Lactobacillus *S64 for 18 h (the ratio of the fluid and S medium at 2× strength = 1:1, vol/vol) and then* Salmonella *Typhimurium DT104 (10^9 CFU/ml) for the remaining days.*

worm. However, the worms treated with the live *Lactobacillus* cells showed no increase in *Salmonella* colonization on day 4 compared with day 2, whereas worms in the other treatment groups did have a significant increase in *Salmonella* burden. On day 4, the level of *Salmonella* colonization in worms treated with live *Lactobacillus* cells was less than 10^5 CFU per worm. In contrast, the worms treated with the *Lactobacillus* ECF or heat-killed cells had a significant higher level of *Salmonella* colonization ($>10^6$ CFU per worm) on the same day. By days 6 and 8, the difference in *Salmonella* colonization was reduced among different treatment groups, and all the worms had a similar level of *Salmonella* colonization, with less than 10^6 CFU per worm. The effect of different culture fractions (ECF and live and heat-killed cells) of isolate CL11 was also examined in parallel during the assays. Although all the culture fractions of isolate CL11 provide no protection, colonization profiles of *Salmonella* in the intestine of CL11-treated worms were similar to those from the intestines of worms treated with different culture fractions of isolate S64 (data not shown).

Effect of dietary treatment with *Lactobacillus*-fermented feed on pig performance. Table 3 shows the impact of different dietary treatments on pig performance. Pigs on the diet fermented with isolates LB1 or S64 had the best growth performance, with the highest ADG and feeding efficiency among all the pig groups. In contrast, pigs on CL11- or LB2-fermented feed had the lowest ADG and feeding efficiency. The growth performance of L3- or ALB2-treated pigs was moderate. There was a correlation between the degree of protection offered by the *Lactobacillus* isolates in the *C. elegans* survival assay and the growth performance of pigs treated with the same isolates in the pig trial (ADG: $r = 0.85$, $P = 0.0334$; F/G: $r = -0.87$, $P = 0.025$). A similar trend also was observed for pigs with diarrhea (diarrhea incidence: $r = -0.64$, $P = 0.168$; diarrhea index: $r = -0.64$, $P = 0.192$). Pigs on the diet fermented with isolate LB1 or S64 had the lowest diarrhea incidence and index among all the groups of pigs. However, pigs on CL11-fermented feed had the most severe diarrhea. Feeding LB1- or S64-fermented feed significantly improved pig performance compared with feeding the basal diet without fermentation, either in solid or liquid form.

DISCUSSION

Ikeda et al. *(18)* used *C. elegans* as an animal model to evaluate the influence of lactic acid bacteria on the longevity of the nematode and on the host defense against *Salmonella* Enteritidis. All tested isolates provided effective protection of the nematode from *Salmonella*-induced death. In the present study, we tested 17 *Lactobacillus* isolates from pig and chicken sources. In our microbiological assay, these isolates were not significantly different in the degree to which they inhibited the growth of *Salmonella* Typhimurium DT104. However, these isolates did differ in their ability to protect *C. elegans* from *Salmonella* Typhimurium DT104–induced death, ranging from full, to partial, to no protection. Six of the isolates that provided different degrees of protection (from full to little protection) were further tested on pigs. The protection trend was similar, affecting pig performance through fermented feed regardless of the source of the isolates. This correlation suggests that *C. elegans* can be used as a prescreening animal model to study the efficacy of probiotics for inhibition of intestinal pathogens. However, the small size of this preliminary pig experiment on growth performance and lack of *Salmonella* challenge makes us cautious in drawing a definitive conclusion. A larger pig trial is required to verify the suitability of *C. elegans* as a model for prescreening probiotics for use in pigs.

In the present study, the two pig *Lactobacillus* isolates, S64 and CL11, had similar levels and trends of colonization in the intestine of *C. elegans,* although these isolates differed in their ability to protect the nematode from the pathogen. Thus, the colonization of the *C. elegans* intestine by *Lactobacillus* isolates alone does not appear to be the major mechanism through which protection from *Salmo-*

TABLE 3. *Effect of dietary treatments on pig performance*

Measurements[a]	Treatment groups[b]							
	Control I	Control II	CL11	L3	LB2	LB1	ALB2	S64
Growth performance								
ADG (g)	354.17 ± 5.20 C	208.33 ± 3.10 D	154.17 ± 5.94 E	343.75 ± 2.32 C	189.24 ± 3.10 DE	441.67 ± 5.97 AB	392.71 ± 10.55 B	466.67 ± 4.64 A
ADFI (g)	872.58 ± 14.09 A	608.94 ± 10.68 C	464.34 ± 4.15 E	516.77 ± 6.75 D	520.31 ± 5.74 D	816.88 ± 10.55 A	579.46 ± 5.00 C	717.62 ± 3.86 B
F/G	2.49 ± 0.05 B	2.76 ± 0.07 AB	3.02 ± 0.20 A	2.40 ± 0.10 B	2.66 ± 0.08 AB	2.01 ± 0.06 C	2.65 ± 0.03 AB	1.53 ± 0.03 D
Diarrhea								
Diarrheal index	0.52 ± 0.04 AB	0.57 ± 0.12 AB	0.77 ± 0.17 A	0.64 ± 0.08 AB	0.48 ± 0.08 B	0.07 ± 0.01 C	0.32 ± 0.07 B	0.14 ± 0.03 C
Diarrheal incidence	19.25 ± 0.87 B	21.15 ± 3.95 AB	27.33 ± 1.97 A	23.2 ± 3.95 AB	26.55 ± 5.08 A	3.55 ± 0.35 D	10.85 ± 1.48 C	5.19 ± 0.53 D

[a] ADG, average daily body weight gain; ADFI, average daily feed intake; F/G, the ratio of feed intake to body weight gain, indicating feeding efficiency. Feed was calculated based on dry weight.
[b] Data expressed as mean ± standard error; $n = 12$. Control I, basal diet in solid form; control II, basal diet in liquid form. For other treatment groups, *Lactobacillus* isolates CL11, L3, LB2, LB1, ALB2, and S64 were used to ferment the basal diet in liquid form. Within a row, means with different letters differ significantly ($P < 0.05$).

nella-induced death was mediated. The main difference between the live cell and other treatments in protecting the nematode occurred between days 2 and 6 (with the highest death rate; Fig. 4A). This effect was coincident with the largest difference in *Salmonella* colonization in the intestine of *C. elegans* between the treatments, which also occurred from day 2 to day 6 during the assay (Fig. 4B). These observations suggest that the protection offered by the *Lactobacillus* isolate may have been mediated through the interference of *Salmonella* colonization. However, because *C. elegans* treated with different culture fractions (ECF and live and heat-killed cells) of CL11 had *Salmonella* colonization profiles similar to those of nematodes treated with S64, regardless of the difference in protection provided by the live and heat-killed cells, it seems unlikely that *Lactobacillus* provided protection was mainly through interference with *Salmonella* colonization. In previous studies, *Salmonella* infection had a pathogenic effect on *C. elegans (4, 21)*. This pathogen can accumulate in and cause distention of the intestinal lumen of *C. elegans*. *Salmonella* infection also can result in apoptosis of germ cells *(1)*. Virulent gene regulators in *Salmonella* play a role in the pathogenic effect on *C. elegans*. In particular, the PhoP/Q two-component regulators, which are required for *Salmonella* survival in macrophages, are also central to lethality in *C. elegans (1, 29, 41)*. The nematode has inducible immune responses that resemble, at least in part, those of the innate immune systems of mammals *(6, 20, 38)*. Although a Toll-like signaling cascade appears to have no substantial role in *C. elegans* innate immunity *(36),* the nematode possesses at least three signaling pathways involved in antimicrobial responses, including (i) a p38-like mitogen-activated protein kinase pathway, (ii) a transforming growth factor β pathway, and (iii) an insulin or insulin-like growth factor 1 pathway *(1, 3, 10, 13, 19, 24, 27, 42)*. It remains to be determined whether the protective effect of *Lactobacillus* observed in the present study resulted from the interruption of *Salmonella* signaling, from the enhancement of *C. elegans* innate immune responses, or from other unknown mechanisms.

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