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Characterization and specificity of probiotics to prevent *salmonella* infection in mice

Ana Andino[1], Nan Zhang[1**], Sandra Diaz-Sanchez[1], Carrie Yard[1], Sean Pendleton[1], and Irene Hanning*[1,2]**

[1]University of Tennessee, Department of Food Science and Technology, Knoxville TN, 37996, USA; [2]University of Tennessee, Department of Genome Sciences and Technology, Knoxville TN, 37996, USA

[*]**Corresponding author:** Irene Hanning, Ph.D, Assistant Professor University of Tennessee, Department of Food Science and Technology, 2605 River Dr., Knoxville TN, 37996

[*]These authors contributed equally to the described work

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ABSTRACT

Background: Probiotic strains of bacteria can prevent *Salmonella* from causing disease by preventing the pathogen from colonizing the intestines. Two strains of probiotics, *Lactobacillus acidophilius* and *Pediococcus spp*, that were obtained from poultry fecal samples have been shown to be efficacious in poultry. The objective of this study was to determine if these strains of probiotics could prevent salmonellosis in a mouse model.

Methods: First, both strains of probiotics were evaluated for *in vitro* efficacy to inhibit the growth of and interfere with virulence gene regulation in *Salmonella enterica*. For *in vivo* efficacy, mice was used which models Typhoid illness. Mice were divided into 2 groups: Control and treatment, *Lactobacillus* and *Pediococcus* (LP; 10^8 Log CFU). Two experiments were conducted. In the first experiment, the mice were treated with *LP* in water for the first two days of the experiment and challenged with *Salmonella* at day three. In the second experiment, the *LP* treatment was given in the water for 10 days and challenge was performed on day 11. In both experiments, at day 20 post-challenge, all mice were sacrificed, intestinal tracts and organs removed and cultured for *Salmonella*.

Results: The probiotic strains inhibited the growth of *Salmonella* and down-regulation of virulence genes was noted, but dependent on the strain of *Salmonella* being evaluated. For the *in vivo* experiment, the probiotics did not afford the mice protection from infection and increasing the length of time the probiotics were administered did not improve the efficacy of the probiotics.

Conclusions: It appears that these strains of probiotic bacteria are effective against *Salmonella in vitro.* However, these isolates did not afford protection from *Salmonella* infection to mice which may be due to host specifity as these isolates were obtained from poultry.

Keywords: *Salmonella,* Probiotic, *Lactobacillus, Pediococcus,* Mice

BACKGROUND

Bacteria, including *Salmonella,* are becoming resistant to antibiotics making treatment more difficult [1]. Furthermore, antibiotics are retroactive and cannot prevent sequelae including Reiter's syndrome and reactive arthritis. Thus, prevention of infection is key to avoiding life long illnesses. Due to the development of antibiotic resistance, alternatives are being sought which include probiotic bacteria and vaccination. With some consumers, "all natural" prevention methods including probiotics have been more popular [2].

Probiotic bacteria provide a number of benefits to the host including protection from pathogenic bacteria [3]. These bacteria protect the host through several mechanisms including competing for nutrients and niches and production of antimicrobial substances [4,5]. Furthermore, there is evidence that probiotic bacteria can interfere with the gene expression pathways of pathogenic bacteria, which could render the pathogen unable to colonize and cause disease [6].

The performance of probiotic strains may differ with usage in different animals because factors such as adherence sites vary between hosts [2]. It is understood that pathogenic bacteria can be host specific such is the case for many zoonotic bacteria including *Salmonella*. However, it has not been clarified if probiotic bacteria are also host specific. Previous research as well as our own, have demonstrated that a mixed culture of two strains of probiotics are effective at preventing *Salmonella* colonization in broiler chicks [7]. Given the proven efficacy of the probiotic strains used in this study and the source (poultry), the objective of this study was to determine their ability to inhibit *Salmonella* using a Typhoid induced mouse model.

METHODS

Bacteria strains and in vitro characterization

One strain of *Lactobacillus acidophilus* and one strain of *Pediococcus spp.* originally obtained from a poultry cecal sample [7] were the two probiotic bacteria evaluated in this work, were cultured individually in De Man, Rogosa and Sharpe broth (MRS; Thermo Scientific, Pittsburgh, PA) and incubated at 37°C for 24h. After incubation, the medium was passed through a 0.45m filter to produce the sterile spent medium. The pH of the medium was adjusted to 6.2 prior to use. For growth inhibition assays, a total of 11 serovars consisting of 15 strains of *S. enterica* were utilized (Table 1).

All *Salmonella* strains were initially cultured on MRS and incubated at 37°C for 24h. After incubation, a loop of bacteria was inoculated into MRS broth and incubated in a shaking water bath at 37°C for 3h. The cultures then were split into 3 equal aliquots and centrifuged at 8000 × *g* for 5 min. The supernatant was discarded and the pellets were resuspended in sterile MRS or

spent medium from *L. acidophilus* or *Pediococcus*. The pH of the suspensions was measured using a pH meter (Denver Instruments, Bohemia, N.Y., U.S.A.) at specific time points (0, 2, 4, 6, 8, 12, 16, 20, and 24 h).

Table 1. A table of the *Salmonella enterica* serovars, the source of the strains and references describing characteristics of the strains utilized in this work.

***Salmonella enterica* serovar**	**Source**	**Reference**
S. Typhimurium DT104	Human infection	1
S. Typhimurium ATCC 23595 (LT2)	Laboratory strain	8
S. Typhimurium ATCC 14028	Laboratory strain	None
S. Enteritidis (WT)	Human infection	None
S. Enteritidis ATCC 13076	Human infection	None
S. Kentucky	Poultry carcass	9
S. Kentucky	Poultry carcass	9
S. Seftenburg	Poultry farm	10
S. Heidelberg	Poultry farm	10
S. Mbandanka	Poultry carcass	11
S. Newport	Poultry carcass	11
S. Bairely	Poultry carcass	11
S. Javana	Poultry Farm	10
S. Montevideo	Swine farm	10
S. Infantis	Poultry Farm	10

For growth curves, triplicate 200 μL aliquots of the cell suspensions were placed into the wells of a 96 well flat bottom plate. The optical density of the suspensions was determined using a plate reader (ELX 800 Universal Plate Reader; Bio-Tek Instruments, Winooski, Vt., U.S.A.) every hour for a 24h time period at 590nm. At the conclusion of the 24h period, viability of the cultures was evaluated by culturing aliquots of the cell suspension on tryptic soy agar (TSA).

Measurement of virulence gene expression

For these experiments, the 3 strains of *Salmonella* Typhimurium listed in Table 1 were used because this serovar causes disease in mice. The suspensions of *Salmonella* were prepared in the sterile spent media produced by the probiotic bacteria as described in the previous section. The expression of *hilA and invA* were measured as we have previously described [12]. Briefly, at specific time points (0, 2, 4, and 24h) an equal volume of RNA protect bacterial reagent (Qiagen, Valenica, Calif., U.S.A.) was added to the wells of the 12-well plate containing the *Salmonella* suspensions. The entire sample was collected into a 2mL microfuge tube and allowed to stand at room temperature for 5 min. Subsequently, total RNA was extracted from the samples with the RNeasy mini kit (Qiagen) as directed by the manufacturer. After extraction, the RNA samples were subjected to a DNase treatment utilizing the Qiagen DNase kit (Qiagen) as directed by the manufacturer. Prior to use in the Real-Time PCR assay, all samples were quantified spectrophotometrically (Nanodrop ND-1000, ThermoScientific, Pittsburgh, Pa., U.S.A.).

All qRT-PCR reactions were performed using the ABI 7100 (Applied Biosystems, Carlsbad,

Calif., U.S.A.). Sequences for the primer sets, *hilA* and *InvA*, were as we have described [12] and synthesized by Integrated DNA technologies (Coralville, Iowa, U.S.A.). For each reaction, a 20µL total volume consisted of 10µL of EXPRESS SYBR Green ERTM qPCR SuperMix with Premixed ROX (Invitrogen, Carlsbad, Calif., U.S.A.), 0.5µL of EXPRESS SuperScript Mix for One-Step SYBR Green ER (Invitrogen), 500nM of each primer, 100ng of RNA template, and water to volume. The qRT-PCR reaction was optimized to the conditions of 50˚C for 5 min for the initial reverse transcriptase step. This was followed by 40 cycles of 95˚C for 15s, 55˚C for 15s, and 68˚C for 20s with fluorescence being measured during the extension phase. Melting curves were conducted subsequently and consisted of 95˚C for 15 s, 60˚C for 20 min increasing by 0.5˚C per min to a final temperature of 95˚C. All reactions were performed independently and in triplicate. Samples were normalized using the 16S rRNA gene as an internal standard. The relative changes (n-fold) in *hilA* expression between the treated and nontreated samples were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen [13].

In vivo experiments

For *in vivo* experiments, after incubation, a 100µl loop of *Lactobacillus acidophilus* and *Pediococcus* were suspended individually into phosphate buffer solution (PBS, Becton, Dickinson and Company, Sparks, MD USA) and vortexed vigorously. The suspensions were standardized to 1.46 at 630 nm by spectrophotometry for a final concentration of 9 log CFU mL^{-1}. The suspended probiotics were provided daily in the drinking water for the mice for a period of three days in the first experiment and for 10 days in the second experiment prior to *Salmonella* challenge.

Animals and Housing

Five to six weeks-old male BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed individually in standard cages. Animal experiments were conducted with an animal care protocol approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. A total of 15 mice were randomly distributed into 2 groups: 1) control; standard rodent chow and no probiotic treatment; and 2) treatment; *Lactobacillus* and *Pediococcus* (LP) and standard rodent chow delivering the treatment in water.

For challenge, a strain of *Salmonella enterica* serovar Typhimurium DT104 was utilized that was initially cultured on tryptic soy agar (TSA, Becton, Dickinson and Company, Sparks, MD USA) and incubated at 37°C for 24h. After incubation, a 10µl loop of culture was inoculated into tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, MD) and incubated in a shaking water bath at 37°C overnight (12 hours). From this culture, 1mL was inoculated into fresh TSB and incubated in a shaking water bath at 37°C for 3h. The culture then was centrifuged at 8,000 x g for 5 min and the supernatant discarded. The culture was washed 3 times by resuspending the pellet in phosphate buffer solution (PBS, Becton, Dickinson and Company, Sparks, MD) and centrifuging. After washing, the culture was finally resuspended in PBS. *Salmonella* suspensions were standardized to 0.15 at 630 nm by spectrophotometry for a final

concentration of 8 log CFU mL^{-1}. The three groups of mice were infected with 0.25 ml of the bacterial suspension (10^8 log CFU mL^{-1}) by gastric gavage.

The treatment was delivered in water for 2 days (Experiment 1) or 10 days (Experiment 2) prior to challenge with *Salmonella*. After challenge with the *Salmonella*, mice droppings were collected daily and cultured for *Salmonella.* If adverse signs of health appeared, the mice were euthanized before schedule and organs were collected. At day 20-post challenge, surviving animals were sacrificed and the heart, lungs, spleen, liver, kidneys, small intestine and ceca were removed and cultured for *Salmonella*.

Fecal samples and organs

Prior to challenge, fecal samples were taken for two days, to ensure that the mice were free of *Salmonella.* Samples were enriched with Tetratrionate broth base (TET, Thermo Fisher Scientific, Remel Products, Lenexa, KS) supplemented with iodine solution (Thermo Fisher Scientific). The enriched samples were incubated at 37°C for 24h and 100 µl of the culture was plated into XLT4 agar which was incubated at 37°C overnight. Fecal samples were collected from mice every day after *Salmonella* challenge and *Salmonella* in the samples were quantified by making 10-fold dilutions in PBS that were then plated on XLT4 agar. The plates were incubated at 37°C for 24h. The organs collected at dissection were aseptically cut, macerated with a sterile dissecting blade and directly stroked and swabbed onto XLT4 agar and incubated at 37°C overnight. After swabbing directly onto the plates, these organs were enriched in TET and plated onto XLT4 as described for the fecal samples.

RESULTS AND DISCUSSION

The probiotic strains utilized in this work have been demonstrated to be effective against S. Enteritidis *in vivo* using a broiler chick model [7]. The *in vitro* results using the *Pediococcus* strain from the present study agreed with these published findings (Table 2), as the *Pediococcus* strain inhibited the growth of all strains of Salmonella that were evaluated. However, the *Lactobacillus* strain had a limited spectrum of activity and did not inhibit growth in 9 of the 16 strains of *Salmonella* (Table 2).

Similar to our results, published work has demonstrated that some probiotic strains have a limited spectrum of activity while other strains were very broadly active against pathogens [14].

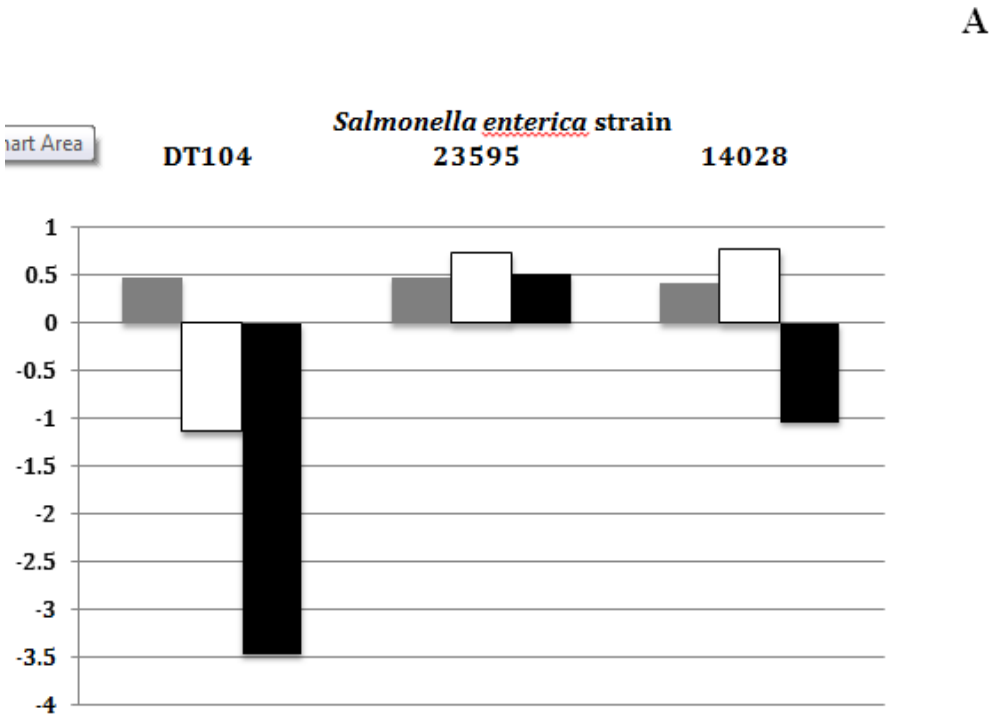
There are several mechanisms of action of the antimicrobial activity of lactic acid bacteria including acid production and bacteriocin production. Production of metabolites by lactic acid bacteria, including acetic and lactic acid, results in an acidic pH and many pathogens including *Salmonella* are sensitive to acidic pH conditions [15]. However, this variable was accounted for by adjusting the pH to nearly neutral prior to suspending the *Salmonella* and thus, pH cannot account for the biocidal activity noted in these experiments. Bacteriocins are also produced by lactic acid bacteria and have antimicrobial activity against many pathogens. Some of these antimicrobials are not sensitive to pH and retain their activity when pH is changed. Therefore, bacteriocins present in the SSM could be responsible for the biostatic activity.

In these experiments, the sterile spent medium from both strains of probiotics down-regulated the expression of *hilA* in the *Salmonella* strain DT104 (Figure 1).

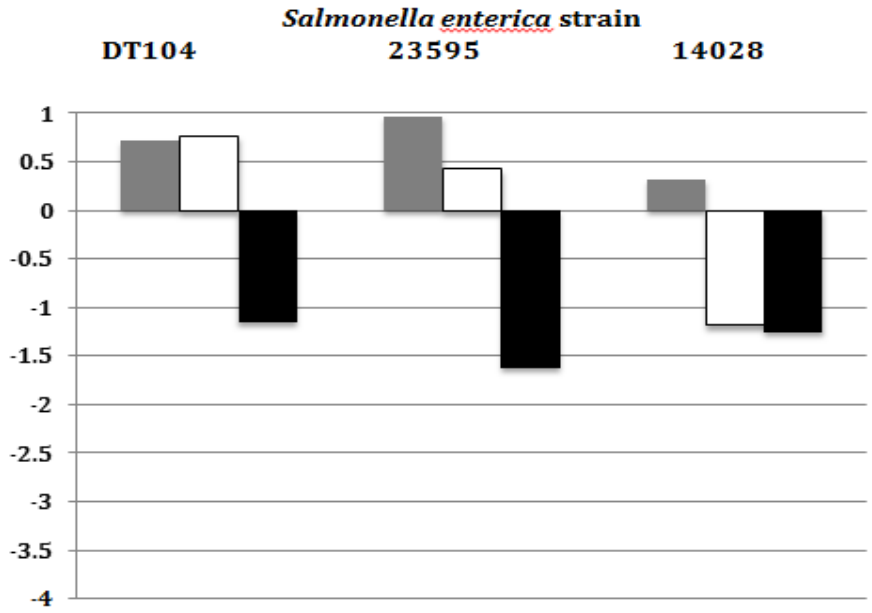
Table 2. Survival of *Salmonella* cultures after 24 h of suspension in sterile spent MRS medium produced by probiotic cultures. The *Salmonella* cultures were suspended in MRS, incubated at 37C and optical density (630nm) measured every hour for 24h.

	Control	***Lactobacillus***	***Pediococcus***
S. Typhimurium DT104	0.965 ±0.02	0.95 ±0.02	0.822±0.05
S. Typhimurium ATCC 23595 (LT2)	0.965 ±0.02	0.934±0.02	0.75±0.02
S. Typhimurium ATCC 14028	0.965 ±0.02	0.683±0.06	0.52±0.03
S. Enteritidis (WT)	0.965 ±0.02	0.965±0.02	0.82±0.08
S. Enteritidis ATCC 13076	0.965 ±0.02	0.86±0.06	0.717±0.02
S. Kentucky	0.965 ±0.02	0.88±0.04	0.72±0.06
S. Kentucky	0.965 ±0.02	0.96±0.02	0.81±0.02
S. Seftenburg	0.965 ±0.02	0.795±0.06	0.664±0.06
S. Heidelberg	0.965 ±0.02	0.93±0.06	0.75±0.03
S. Mbandanka	0.965 ±0.02	0.96±0.06	0.66±0.02
S. Newport	0.965 ±0.02	0.98±0.02	0.79±0.09
S. Bairely	0.965 ±0.02	0.99±0.03	0.762±0.06
S. Javana	0.965 ±0.02	0.864±0.06	0.754±0.03
S. Montevideo	0.965 ±0.02	0.866±0.06	0.7±0.06
S. Infantis	0.965 ±0.02	0.95±0.06	0.795±0.03

Figure 1. Fold change in regulation of *hilA* (A) and *InvA (*B*)* virulence genes in *Salmonella enterica* serovar Typhimurium after suspension in sterile spent medium produced by *Pediococcus spp.* or *Lactobacillus acidophilus*.



B



However, down-regulation of *invA* was either absent or not as signficant in the same strain of *Salmonella*. These findings are important because when *Salmonella* encounters the gastrointestinal environment, transcription of these genes may be activated [16]. Thus methods which interfere with regulation of these genes can effectively inhibit colonization. An acidic pH has been demonstrated to supress *hilA* and *invA* [17]. Because the pH of the SSM was adjusted and like biostatic activity, pH cannot account for suppression of these genes. A possible explanation for the down-regulation may be attriubted to bacteriocins present in the SSM. These antimicrobials could have initiated a stress response and therefore, energy efforts were shifted away from virulence and allocated towards survival genes [12].

The probiotic strains utilized in this research did not afford the mice any protection (Table 3,4). Several mechanism failures may explain the lack of efficacy. First, Letellier et al. [18] suggested that to be effective in excluding pathogen infection, a massive colonization of the intestinal tract by the probiotic bacteria is required. For this reason, the probiotics used in these experiments, may not have colonized to sufficient concentrations to prevent infection. Secondly, disruptions to the normal microflora may leave the host more susceptible to infection [19]. This may explain why probiotics do not persist after administration is discontinued, as well as the failure of long-term changes in the intestinal microbiota using probiotics. In Experiment 1, we administered the *Lactobacillus* and *Pediococcus* cultures twice prior to challenge. Challenge was delivered 24 hours after the probiotics were removed. Thus, it is likely that the host bacterial profile had not returned in this short amount of time and instead the probiotics did not afford the mice protection from infection for other reasons.

The performance of probiotic bacterial strains differs because different bacteria have defined adherence sites, immunological effects, and varied effects in the healthy versus inflamed mucosal milieu [2]. In a previous study by Gueimonde *et al*. [4], 3 strains of *Lactobacillus casei* were evaluated and the authors reported that the strain TMC 0409 was the most effective strain

for inhibiting the adhesion of *Salmonella* Typhimurium ATCC 29631. The authors concluded that the inhibition was related to specific adhesives and receptors for which probiotics and pathogens are competing [20]. Additionally, Perdigon *et al*. [21] demonstrated of 3 probiotic bacteria, *Lactobacillus casei* and *Lactobacillus bulgaricus* were able to activate macrophages in mice and suggest that these bacteria, when passing through the intestinal tract, may be responsible for the enhanced host immune response. Given these studies, it may be that the probiotic bacteria evaluated in these experiments were not as effective in activating immune cells. Furthermore, it is also possible that the probiotic bacteria used in these experiments were not as specific to the epithelia receptors as other strains have been demonstrated to be [20].

Typically, *S.* Typhimurium in a mouse model will translocate across the intestinal tract becoming systemic infecting many of the organs. Furthermore, *Salmonella* persists for as long as 30 days post-inoculation, infecting organs but absent from the gastrointestinal tract [19] In this study, the results from Experiment 1 support these statements (Table 3). However, it appears that in Experiment 2, *Salmonella* was colonizing the intestinal tract as culturing recovered *Salmonella* from both fecal samples and intestinal samples (Table 4). The reason for this difference is unclear because the mice were given the same challenge dosage of *Salmonella* in both experiment.

Table 3. Detection of *Salmonella* Typhimurium LT2 in mouse fecal samples and organs after necropsy. Mice were administered probiotic bacteria in the water (*Lactobacillus* and *Pediococcus*) for 2 days. At day 3, all mice were challenged with *Salmonella* Typhimurium DT104. Control group (C) and *Lactobacillus* / *Pediococcus* (LP).

	Day Number Post-Salmonella Challenge																			
Mouse	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Necropsy
C1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.7	ND	ND	2.3	ND	ND	ND	ND	ND
C2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S, LV
C3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S
C4	2.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	C
C5	2.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S, C
LP1	5.171	DEAD																			H, L, Lv, S, K, C
LP2	ND	ND	ND	ND	ND	ND	ND	ND	DEAD												H, L, Lv, S, K, C
LP3	ND	ND	ND	ND	ND	ND	ND	ND	DEAD												H, L, Lv, S, K, C
LP4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.3	ND	ND	ND	ND	2.2	ND	ND	DEAD		H, L, Lv, S, K, C
LP5	2.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S

[1]ND=Not detected; D=Detected after enrichment of fecal material in Tetrathionate Broth (TET).

[2] Mice that died prior to the end of the experiment were necropsied immediately after death and infected organs are listed in last column. H=heart, L=Lungs, L=Liver, S=Spleen, K=Kidney, C=Cecum, In=small intestine

Table 4. Detection of *Salmonella* Typhimurium DT104 in mouse fecal samples and organs after necropsy. Mice were administered probiotic bacteria in the water (*Lactobacillus* and *Pediococcus*) for 10 days. At day 11, all mice were challenged with *Salmonella* Typhimurium DT104. Control group (C) and *Lactobacillus* / *Pediococcus* (LP).

Day Number Post-*Salmonella* Challenge

Mouse	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Necropsy
C1	ND[1]	ND	ND	5.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	DEAD[2]									H, L, Lv, S, K, In
C3	ND	ND	ND	ND	ND	ND	ND	ND	D	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C4	ND	ND	ND	ND	D	D	ND	DEAD													L, S, In
C5	D[1]	ND	ND	ND	D	D	ND	3.44	DEAD												H, L, Lv, S, K, In
LP1	D	ND	D	D	ND	ND	ND	DEAD													H, L, Lv, S, K, In
LP2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LP3	D	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LP4	D	2.4	2.4	2.1	DEAD																H, L, Lv, S, K, In
LP5	ND	ND	ND	ND	ND	3.6	D	DEAD													L, S, In

[1] ND=Not detected

[2] Mice that died prior to the end of the experiment were necropsied immediately after death and infected organs are listed in last column. H=heart, L=Lungs, L=Liver, S=Spleen, K=Kidney, C=Cecum, In=small intestine

CONCLUSIONS

In conclusion, it appears that the probiotic strains used in these experiments had biostatic activity in vitro, but did not protect mice from *Salmonella* infection *in vivo*. Published studies indicate that the reasons may be because there was not a sufficient concentration of probiotic bacteria in the intestinal tract and the specificity to epithelial receptors may not have been ideal given that the source of these probiotics were from poultry fecal samples. Additionally, the length of time between probiotic administration and *Salmonella* challenge may have been too short to allow activation of the immune system in a sufficient manner to enhance protection against infection.

Competing interests: The authors declare no competing interests.

Authors contributions: AA, NZ, SDS, CY and SP conducted the in vivo experiments. NZ conducted the in vitro experiments. AA, NZ, SDS and IH analyzed and interpreted the data. IH prepared the manuscript. All authors reviewed the manuscript.

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