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Selected Lactic Acid-Producing Bacterial Isolates with the Capacity to Reduce *Salmonella* Translocation and Virulence Gene Expression in Chickens

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Abstract

Background: Probiotics have been used to control *Salmonella* colonization/infection in chickens. Yet the mechanisms of probiotic effects are not fully understood. This study has characterized our previously-selected lactic acid-producing bacterial (LAB) isolates for controlling *Salmonella* infection in chickens, particularly the mechanism underlying the control.

Methodology/Principal Findings: *In vitro* studies were conducted to characterize 14 LAB isolates for their tolerance to low pH (2.0) and high bile salt (0.3–1.5%) and susceptibility to antibiotics. Three chicken infection trials were subsequently carried out to evaluate four of the isolates for reducing the burden of *Salmonella enterica* serovar Typhimurium in the broiler cecum. Chicks were gavaged with LAB cultures (10^{6-7} CFU/chick) or phosphate-buffered saline (PBS) at 1 day of age followed by *Salmonella* challenge (10^4 CFU/chick) next day. Samples of cecal digesta, spleen, and liver were examined for *Salmonella* counts on days 1, 3, or 4 post-challenge. *Salmonella* in the cecum from Trial 3 was also assessed for the expression of ten virulence genes located in its pathogenicity island-1 (SPI-1). These genes play a role in *Salmonella* intestinal invasion. Tested LAB isolates (individuals or mixed cultures) were unable to lower *Salmonella* burden in the chicken cecum, but able to attenuate *Salmonella* infection in the spleen and liver. The LAB treatments also reduced almost all SPI-1 virulence gene expression (9 out of 10) in the chicken cecum, particularly at the low dose. *In vitro* treatment with the extracellular culture fluid from a LAB culture also down-regulated most SPI-1 virulence gene expression.

Conclusions/Significance: The possible correlation between attenuation of *Salmonella* infection in the chicken spleen and liver and reduction of *Salmonella* SPI-1 virulence gene expression in the chicken cecum by LAB isolates is a new observation. Suppression of *Salmonella* virulence gene expression *in vivo* can be one of the strategies for controlling *Salmonella* infection in chickens.

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Introduction

Salmonella is a zoonotic pathogen. Its infection in human and domestic animals remains a serious and worldwide issue in public health, food safety, and livestock economies [1]. Consumption of *Salmonella*-contaminated livestock and their products, particularly poultry and poultry products, has often led to salmonellosis in humans. In addition, the increased level of antibiotic resistance in *Salmonella* recovered from animals potentially erodes the therapeutic effectiveness in antibiotic treatment of animal and human disease. Thus, control of *Salmonella* infection in animals is still urgently required.

Salmonella enterica serovar Typhimurium is one of three serotypes, sometimes responsible for 50% of the salmonellosis cases in humans [2]. The susceptibility of humans and animals to *Salmonella* infection is generally determined by both *Salmonella* virulence and host factors, such as age and genetics. The infection normally starts from the intestinal phase, through which *Salmonella* can enter the blood circulation and spread to various organs such as the liver and spleen, leading to systemic infection. There are two pathogenicity islands in *Salmonella* that are closely associated with virulence [3]. *Salmonella* pathogenicity island 1 (SPI-1) is critical in causing the intestinal phase of infection [3], while *Salmonella* pathogenicity island 2 (SPI-2) is essential for intracellular multiplication, which is necessary for systemic infection [4]. The SPI-1 is composed of a 40-kb DNA region coding for the type III secretion system (T3SS), including the structural, effector, and regulatory proteins.

It is important to control *Salmonella* infection at the farm level in order to reduce *Salmonella* contamination in poultry and poultry products. Attempts to control *Salmonella* infection and carriage by

vaccination and antibiotic treatment of chickens have not yet produced significant results [5,6]. An alternative approach has been the use of competitive exclusion and probiotics [7]. Competitive exclusion of *Salmonella* is achieved by enhancing the establishment of normal intestinal microbiota in newly hatched chickens and thus inhibiting intestinal colonization of *Salmonella* [8]. Probiotics can have different modes of action to control the infection of enteric pathogens, such as secretion of antimicrobial substances (for example bacteriocins) [9], enhancement of digestive functions [10], and modulation of the mucosal and systemic immune responses [11,12]. Control of *Salmonella* burden and colonization has been one of the common strategies. Previous studies have shown that the expression of gene encoding virulence factors of *Salmonella* can be modulated by probiotics *in vitro* [13,14,15,16,17]. Recently, we identified several lactic acid-producing bacterial (LAB) isolates that were able to protect *Caenorhabditis elegans* from death caused by *S.* Typhimurium infection [18]. In the present study, we have investigated the probiotic properties of these isolates and, moreover, if they are able to reduce *Salmonella* infection in broilers and also modulate *Salmonella* SPI-1 virulence gene expression.

Results

Resistance of selected lactic acid-producing bacterial isolates to low pH, high bile salt, and antibiotics determined by *in vitro* studies

Among the 14 tested LAB isolates, eight (ALB2, ALB6, CL9, L3, LB1, LB2, LB4, and S64) have been reported previously to have antimicrobial activity towards *S.* Typhimurium DT104 [18]. The remaining six isolates (K16, K67, S8, S33, S66, and SG2) were also tested for their antimicrobial activity using the method of spot-on-the-lawn [19]. These isolates were able to inhibit the growth of *S.* Typhimurium DT104 with clear inhibition halos (diameter $\geq$16 mm).

Table 1 shows the tolerance of 14 selected LAB isolates to low pH pretreatment. Six isolates (ALB2, CL9, L3, LB1, LB4, and SG2) had similar optical densities at 600 nm (OD_{600}) after growth regardless if the cultures had been pretreated at pH 2 or 5.6, indicating their tolerance to low pH. However, the growth of seven out of eight remaining isolates (K16, K67, LB2, S8, S33, S64, and S66) was inhibited by the low pH pretreatment ($P<0.05$). In the test to examine tolerance to high bile salt, four (ALB2, CL9, K16, and S33) out of the 14 isolates showed poor growth ($OD_{600}<0.5$) after 24 h incubation at 37°C at most concentrations of bile salt above 0.3% (v/v), suggesting that they are sensitive to bile acid (Table 2). Eight isolates (K67, L3, LB1, LB2, LB4, S8, S66, and SG2) were able to grow with OD_{600} values greater than 1.0 at concentrations of bile salt from 0.3% to 1.5% (v/v), indicating good tolerance to bile salt. The remaining two isolates (ALB6 and S64) had a moderate growth with the OD_{600} values between 0.5 and 1.0 after 24 h incubation at bile salt concentrations above 0.3% (v/v).

Table 3 shows the susceptibility of the 14 tested LAB isolates to eight different antibiotics. The isolates varied in their susceptibility to the antibiotics. Three isolates (LB1, LB2, and LB4) were susceptible to all tested antibiotics within the concentrations examined. The majority of remaining isolates grew at the highest concentration of one or two antibiotics. Based on the microbiological cut-off values set by the European Food Safety Authority [20] and the European Commission [21], the majority of the 14 isolates could be categorized as susceptible to ampicillin and erythromycin and about half of the isolates were resistant to gentamicin, tetracycline, and ciprofloxacin.

Effect of selected lactic acid-producing bacterial isolates on *Salmonella* burden in the cecum of chickens

Isolates LB1 (*Lactobacillus zeae*), S8 (*Lactobacillus plantarum*), S64 (*Lactobacillus reuteri*), and S33 (*Pediococcus pentosaceus*) were selected for further *in vivo* studies after the *in vitro* characterization. The selection was based on their resistance to low pH, high bile salt, and antibiotics, their species identification, and previously reported ability to protect *C. elegans* from death caused by *Salmonella* infection [18]. In the *in vivo* studies, cecal digesta samples from all negative control groups of chickens (three chicken trials) had undetectable *Salmonella* counts that were below the detection limit of the plating method (2×10^2 Colony Forming Units (CFU)/g digesta) [22]. In Trial 1, *Salmonella* counts in the cecal digesta from the chickens in the positive control group (challenged with *Salmonella* only) on day 4 post-infection (PI) were 5.33 ± 0.13 (Log_{10} CFU/g fresh digesta). LB1, S8, S33, or S64 treatment groups had 5.70 ± 0.12, 5.38 ± 0.12, 5.47 ± 0.13, or 5.78 ± 0.07 Log_{10}CFU of *Salmonella*/g fresh digesta in their ceca. No significant difference among these five treatments ($P>0.05$) was found.

Since the experimental design of Trials 2 and 3 was identical, data from these two trials were analyzed by a mixed model with trial as a fixed effect (Table 4). When the data from Trials 2 and 3 were pooled, among the four probiotic treatment groups, the low and high dose mixed culture groups and the high dose LB1 group had significantly higher *Salmonella* counts in the cecal digesta on days 3 and 4 PI than the positive control, indicating that the selected individual probiotic candidates (LB1, S8, and S64) or their combination at the tested level could not reduce the *Salmonella* burden in the cecum of chickens.

Effect of selected lactic acid-producing bacterial isolates on invasion of *Salmonella* in the spleen and liver of chickens

In Trial 1, eight and seven birds out of the 12 birds from the positive control group were found to be *Salmonella* positive in the spleen or liver, respectively, on day 4 PI after the tissue samples were enriched for *Salmonella*. This suggests a successful *Salmonella* challenge at the dose of 1×10^4 CFU/chick. As shown in Tables 5 and 6, the data on spleen and liver infection from Trials 2 and 3 were combined for analyses with trial as a fixed effect. No significant effect ($P>0.05$) of trial was observed. Compared with the positive control, the treatment with a low dose of mixed LAB cultures was able to attenuate *Salmonella* infection in the spleen (73% vs. 32%, $P=0.06$) and liver (74% vs. 44%, $P=0.07$) on days 3 and 4 PI. A similar result was also observed with the treatment of low dose LB1, which reduced *Salmonella* infection in the liver (74% vs. 47%, $P=0.09$).

Effect of selected lactic acid-producing bacterial isolates on the SPI-1 virulence gene expression of *Salmonella* in the cecum of chickens

In Trial 3, expression of 10 SPI-1 virulence genes (*hilA, hilC, hilD, sopB, sopD, sopE2, sipA, sipC, avrA*, and *sptP*) of *Salmonella* in the cecal digesta of chickens was investigated by RT-qPCR assays. Compared with the *Salmonella* cells from the chickens in the positive control group, application of LB1 or mixed LAB cultures did not significantly ($P>0.05$) affect expression levels of the 10 virulence genes on day 1 PI. Interestingly, expression of all the genes except *hilC* in the *Salmonella* cells from all the LAB treatment groups of chickens, regardless of dose and combinations, was down-regulated by 4 to 257 fold ($P<0.05$) on day 3 PI compared with the positive control group (Table 7). The low dose group

Table 1. Growth (OD_{600}) of selected lactic acid-producing bacterial isolateswith or without pretreatment of low pH*.

	Optical density at 600 nm (OD_{600})	
Isolate#	**pH 5.6**	**pH 2.0**
ALB2	0.99 [a]	0.98 [a]
ALB6	0.85 [b]	0.88 [a]
CL9	1.00 [a]	1.01 [a]
K16	0.97 [a]	0.87 [b]
K67	1.07 [a]	0.89 [b]
L3	1.05 [a]	1.03 [a]
LB1	1.01 [a]	1.03 [a]
LB2	1.02 [a]	0.58 [b]
LB4	1.01 [a]	1.02 [a]
S8	1.04 [a]	0.75 [b]
S33	1.12 [a]	0.95 [b]
S64	0.93 [a]	0.72 [b]
S66	1.06 [a]	0.73 [b]
SG2	0.73 [a]	0.74 [a]

*Tolerance to low pH was evaluated by incubating bacterial cells in simulated gastric fluid (pH 2.0 or 5.6) for 2 h prior to anaerobic incubation in the MRS broth at 37°C for 20 h.Values without common superscript in a row are significantly different ($P<0.05$). Values were average of triplicates.

#The 14 selected lactic acid-producing bacterial isolates belong to *Lactobacillus crispatus* (SG2), *Lactobacillus plantarum* (S8 and S66), *Lactobacillus salivarius* (ALB2 and ALB6), *Lactobacillus reuteri* (CL9, K67, and S64), *Lactobacillus rhamnosus* (L3, LB2, and LB4), *Lactobacillus zeae* (LB1), and *Pediococcus pentosaceus*(K16 and S33), based on the sequence analysis of 16S rRNA genes. While isolates ALB2, ALB6, L3, LB1, LB2, LB4, and SG2are from chickens, isolates CL9, K16, K67, S8, S33, S64, and S66 have the pig origin.

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treated with mixed LAB cultures had the most dramatic decrease (18 to 257 fold) for all the genes, followed by the low dose LB1 group. Nevertheless, there was no significant ($P>0.05$) difference in the gene expression of *Salmonella* from these four groups of chickens that had been treated with LAB isolates. *In vitro* expression of SPI-1 virulence genes was also examined. Nine out of the 10 virulence genes in *Salmonella* were down-regulated when co-cultured with the extracellular culture fluid (ECF) from isolate LB1 cultures (Table 7). Only *sipC* was up-regulated.

Discussion

This study was to characterize our recently identified LAB isolates for controlling *Salmonella* infection in broilers, including

Table 2. Growth (OD_{600}) of selected lactic acid-producing bacterial isolates at different concentrations of bile salt*.

	Bile salt concentrations (%)				
	0	**0.3**	**0.6**	**1.0**	**1.5**
ALB2	1.96^{a}	0.90^{b}	0.43^{c}	0.31^{c}	0.31^{c}
ALB6	1.93^{a}	1.16^{b}	0.59^{c}	0.65^{bc}	0.65^{bc}
CL9	1.86^{a}	0.08^{b}	0.04^{b}	0.09^{b}	0.21^{b}
K16	1.86^{a}	0.21^{b}	0.05^{b}	0.16^{b}	0.19^{b}
K67	2.02^{a}	1.03^{b}	1.13^{ab}	1.33^{ab}	1.53^{ab}
L3	2.10^{a}	1.97^{a}	2.07^{a}	2.13^{a}	2.06^{a}
LB1	2.06^{a}	1.83^{a}	1.97^{a}	1.94^{a}	1.95^{a}
LB2	1.96^{a}	1.70^{b}	1.30^{c}	1.21^{d}	1.17^{d}
LB4	1.97^{a}	1.68^{b}	1.28^{c}	1.25^{cd}	1.20^{d}
S8	1.98^{a}	1.14^{a}	1.24^{a}	0.87^{a}	1.40^{a}
S33	1.92^{a}	0.28^{b}	0.15^{b}	0.49^{b}	0.86^{ab}
S64	1.82^{a}	1.12^{b}	0.58^{b}	0.79^{b}	0.98^{b}
S66	2.04^{a}	1.73^{b}	1.99^{a}	2.13^{a}	2.12^{a}
SG2	1.93^{a}	1.20^{a}	1.16^{a}	1.52^{a}	1.57^{a}

*Optical density at 600 nm (OD_{600}) was measured after incubation in the MRS broth at 37°C for 24 h under anaerobic atmosphere. The level of each inoculum was 1%. Values were average of triplicates. Values without common superscript in a row are significantly different ($P<0.05$).

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Table 3. Susceptibility of selected lactic acid-producing bacterial isolates to various antibiotics.

Antibiotic	MIC (μg/ml) or inhibitory zone (mm)*													
	ALB2	ALB6	CL9	K16	K67	L3	LB1	LB2	LB4	S8	S33	S64	S66	SG2
Penicillin G	0.3	0.3	2	2	0.5	0.8	1	1.3	0.8	21.3	0.2	1.7	0.8	1
(0–256 μg/ml)														
Ciprofloxacin#	13.3^{R}	4^{S}	$>32^{R}$	$>32^{R}$	$>32^{R}$	1^{S}	4^{S}	2^{S}	2^{S}	$>32^{R}$	$>32^{R}$	5.3^{R}	$>32^{R}$	$>32^{R}$
(0–32 μg/ml)														
Tetracycline#	$>256^{R}$	$>256^{R}$	$>256^{R}$	64^{R}	42.7^{R}	0.5^{S}	0.5^{S}	1.7^{S}	0.7^{S}	16^{S}	16^{S}	32^{R}	16^{S}	$>256^{R}$
(0–256 μg/ml)														
Erythromycin#	0.5^{S}	0.7^{S}	1^{S}	1^{S}	$>256^{R}$	0.2^{S}	0.5^{S}	0.3^{S}	0.3^{S}	1^{S}	0.5^{S}	$>256^{R}$	0.7^{S}	32^{R}
(0–256 μg/ml)														
Ampicillin#	0.5^{S}	0.5^{S}	1^{S}	4^{S}	0.3^{S}	12^{R}	2^{S}	4^{S}	2^{S}	1.5^{S}	0.3^{S}	1^{S}	0.3^{S}	1^{S}
(0–256 μg/ml)														
Gentamicin#	32^{R}	21.3^{R}	16^{S}	64^{R}	16^{R}	10.7^{S}	13.3^{S}	16^{S}	8^{S}	32^{R}	16^{S}	13.3^{R}	8^{S}	32^{R}
(0–256 μg/ml)														
Chloramphenicol	22	26.4	15.3	20.5	23.7	22.7	21.3	20	21.8	14. 7	22.8	25	22.5	14.2
(30 μg/ml)														
Lincomycin	0	0	0.6	0	0	11.8	15	13.9	10.7	0	0	0	15	10
(2 μg/ml)														

*MIC (Minimum Inhibitory Concentration, μg/ml) for penicillin G, ciprofloxacin, tetracycline, erythromycin, ampicillin, and gentamicin was evaluated on the MRSagar with MIC Evaluator Strips (Oxoid) through gradient diffusion tests. The inhibitory zone (mm) for chloramphenicol and lincomycin was determined on the MRS agar using Oxoid Antimicrobial Discs with the concentrations indicated in the table. Values were average of triplicates.
#S, susceptible; R, resistant. Resistance is based on European Food Safety Authority [20] and European Commission [21].
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Table 4. *Salmonella* counts in the cecal digesta on days 3 and 4 post-infection in Trials 2 and 3*.

Treatment	Log_{10}CFU/g digesta (mean ± standard error)			*P* value#		
	Trial 2 (n = 14)	Trial 3 (n = 8)	Combined (n = 22)	Trial 2	Trial 3	Combined
Negative control	0	0	0	NA	NA	NA
Positive control	5.36±0.14	6.58±0.23	5.80±0.17	NA	NA	NA
Low dose LB1	5.18±0.45	5.82±0.41	5.41±0.32	0.95	0.08	0.18
High dose LB1	6.43±0.16	7.04±0.07	6.65±0.12	<0.01	0.44	<0.01
Low dose mixture	6.98±0.06	6.77±0.12	6.90±0.06	< 0.01	0.94	< 0.01
High dose mixture	6.94±0.05	6.86±0.17	6.91±0.07	<0.01	0.84	<0.01

*Aftera series of 1:10 dilution, cecal digesta samples were incubated at 37°C for 24 h on Brilliant Green Sulfa Agar (BGS) supplemented with 200 μg/ml nalidixic acid.
#Individual trial was analyzed by SAS GLIMMIX procedure and Dunnett's method was used for adjustment of multiple comparisons with the positive control group as the control. The negative control group was not included for multiple comparison analysis. SAS GLIMMIX procedure was used for analysis of the two trials simultaneously (combination of the two trials) with trial as a fixed effect. NA, not applicable; *P* value for trial effect <0.01.
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determination of the mechanism for the control in particular. We have demonstrated that orally administered LAB isolates at a low dose (10^6 CFU/strain/chick) could attenuate *Salmonella* infection in the spleen and liver of broilers, which coincided with down-regulation of *Salmonella* SPI-1 virulence gene expression in the chicken cecum. In addition, our data on *in vitro* expression of *Salmonella* SPI-1 virulence genes also support our *in vivo* observations, suggesting a possible correlation between the attenuation of organ infection and down regulation of SPI-1 virulence gene expression. The LAB treatments were unable to lower cecal *Salmonella* burden in the present study. The reduction of *Salmonella* burden in the gastrointestinal tract by probiotics is subjected to multiple factors, among which antimicrobial substances (*e.g.* bacteriocins) produced by probiotic bacteria can have a direct impact [9]. In this regards, we speculate that the LAB isolates tested in the present study may not have produced antimicrobial substances. Further studies are required to determine the underlying reason.

S. Typhimurium harbours more than 80 virulence genes. The ten genes examined in the present study are located in SPI-1 and have important roles in the intestinal invasion of *Salmonella* [4]. Expression of SPI-1 genes is controlled by 5 regulators (HilA, HilC, HilD, InvF, and SprB) with HilA being a master regulator [4]. In addition, these SPI-1 regulators can modulate SPI-2 gene expression that is essential for intracellular multiplication and lead to systemic infections. For instance, HilD is required for activating

Table 5. *Salmonella* invasion to the spleen on days 3 and 4 post-infection*.

Treatment	Infected birds/total birds (infection percentage, %)			*P* value#		
	Trial 2	Trial 3	Combined	Trial 2	Trial 3	Combined
Negative control	0/8	0/14	0/22	0.03	<0.01	<0.01
	(0)	(0)	(0)			
Positive control	5/8	11/14	16/22	NA	NA	NA
	(63)	(79)	(73)			
Low dose LB1	3/8	7/14	10/22	0.62	0.24	0.14
	(38)	(50)	(45)			
High dose LB1	3/8	9/14	12/22	0.62	0.68	0.28
	(38)	(64)	(55)			
Low dose mixture	1/8	6/14	7/22	0.12	0.12	0.06
	(13)	(43)	(32)			
High dose mixture	7/8	10/14	17/22	0.57	1.00	0.74
	(88)	(71)	(77)			

*Samples were enriched overnight at 37°C in Selenite Brilliant Green Sulfa Enrichment (Difco) broth, followed by plating onto Brilliant Green Sulfa Agar (BGS) supplemented with 200 μg/ml nalidixic acid to determine the presence or absence of *Salmonella* colonies.
#Individual trial was analyzed by exact logistic regression via SAS LOGISTIC procedure. SAS GLIMMIX procedure was used for analysis of the two trials simultaneously (combination of the two trials) with trial as a fixed effect, except that, for groups with zero (*i.e.* the negative control), exact logistic regression was used with trial as stratum via SAS LOGISTIC procedure. NA, not applicable; *P* value for trial effect = 0.21.
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Table 6. *Salmonella* invasion to the liver on days 3 and 4 post-infection*.

Treatment	Infected birds/total birds (infection percentage, %)			*P* value#		
	Trial 2	Trial 3	Combined	Trial 2	Trial 3	Combined
Negative control	0/14	0/20	0/34	<0.01	<0.01	<0.01
	(0)	(0)	(0)			
Positive control	8/14	17/20	25/34	NA	NA	NA
	(57)	(85)	(74)			
Low dose LB1	5/14	11/20	16/34	0.45	0.08	0.09
	(36)	(55)	(47)			
High dose LB1	9/14	14/20	23/34	1.00	0.45	0.62
	(64)	(70)	(68)			
Low dose mixture	3/14	12/20	15/34	0.12	0.16	0.07
	(21)	(60)	(44)			
High dose mixture	13/14	16/20	29/34	0.08	1.00	0.30
	(93)	(80)	(85)			

*Samples were enriched overnight at 37°C in Selenite Brilliant Green Sulfa Enrichment (Difco) broth, followed by plating onto Brilliant Green Sulfa Agar (BGS) supplemented with 200 μg/ml nalidixic acid to determine the presence or absence of *Salmonella* colonies.

#Individual trial was analyzed by exact logistic regression via SAS LOGISTIC procedure. SAS GLIMMIX procedure was used for analysis of the two trials simultaneously (combination of the two trials) with trial as a fixed effect, except that, for groups with zero (*i.e.* the negative control), exact logistic regression was used with trial as stratum via SAS LOGISTIC procedure.NA, not applied; *P* value for trial effect = 0.09.

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the regulatory region of the *ssrAB* operon, a key regulator of SPI-2 genes [4,23]. In the present study, we have examined the gene expression of *hilA*, *hilC*, and *hilD* in response to LAB treatment. The expression of *hilA* and *hilD* genes was significantly down-regulated by both single and mixed LAB isolates, particularly at the low dose. Translocation of the SPI-1 effectors (AvrA, SipA, SipC, SopB, SopD, SopE2, and SptP) into the host enterocytes via the type III secretion system (T3SS) is responsible, at least in part, for the intestinal invasion [24,25,26]. AvrA, a member of a family of acetyl transfereases, can dampen host inflammatory and

Table 7. Fold decrease in virulence gene expression of *Salmonella in vivo* and *in vitro*[a].

Gene	*In vivo*				*In vitro*
	Low dose LB1	High dose LB1	Low dose mixture	High dose mixture	LB1-ECF
	(n = 3)	(n = 6)	(n = 6)	(n = 6)	(n = 3)
hilA	15.99±8.95 *	8.03±2.43*	29.29±16.99*	14.54±7.16*	3.95±0.49 b#
hilC	7.02±2.42	1.07±0.20	24.72±7.02	4.47±1.63	3.69±0.40#
hilD	114.38±47.73 *	23.72±4.71*	256.61±56.91*	32.20±14.66*	1.84±0.15#
sopB	26.77±13.06*	13.10±2.84*	82.65±42.89*	45.60±18.21*	3.17±0.56#
sopD	8.20±5.01*	4.64±0.93*	18.05±6.62*	17.29±6.83*	3.29±0.65#
sopE2	41.19±20.26*	58.30±15.2*	186.37±61.57*	35.57±15.05*	4.13±0.40#
sipA	27.88±9.43*	7.48±1.32*	215.38±61.24*	28.21±8.16*	4.61±0.57#
sipC	27.72±9.36*	12.18±2.19*	156.46±43.54*	15.84±4.13*	**9.84±1.46**#
avrA	13.73±5.42*	3.80±0.87*	49.97±12.66*	6.04±1.26*	5.99±0.64#
sptP	20.15±6.85*	13.37±2.88*	87.08±27.48*	16.86±5.55*	1.60±0.52#

[a]*In vivo* gene expression: *Salmonella* cells were from the cecum of the chickens on day 3 post-infection after LAB treatments; *in vitro* gene expression: *Salmonella* cells were from the cultures that had been co-cultured for 14 h with the extracellular culture fluid (ECF) from 16 h-grown *L. zeae*LB1 cultures. Values were expressed as mean ± standard error. The fold-change of target gene was analyzed using the $2^{-\Delta\Delta Ct}$ method. The values derived from $2^{-\Delta\Delta Ct}$ represent fold changes of samples in the abundance relative to the reference groups. In the *in vivo* experiment, the reference group was the positive control chicken group (n = 5) that had been treated with *Salmonella* only. In the *in vitro* experiment, the reference samples were the *Salmonella* cultures (n = 3) that had been treated with the MRS medium instead of the ECF from *L. zeae*LB1 cultures. The reference samples in both *in vivo* and *in vitro* experiments have the $2^{-\Delta\Delta Ct}$ value of 1.

*Values in the same row indicate significant differences ($P<0.05$) compared with the positive control chicken group in the *in vivo* experiment. Values represent down-regulation (fold-decrease) of gene expression.

#Value for each gene represents a significant difference ($P<0.05$) between the treatment and control samples in the *in vitro* experiment. Values represent down-regulation (fold-decrease) of gene expression except for the value in bold that was up-regulated (fold-increase).

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apoptotic defense [27,28]. SipA affects actin cytoskeleton, induces membrane shuffling, and promotes inflammation of the host cells. SipC is involved in actin bundling and nucleation. Through its phosphoinositide phosphatase activity, SopB participates in a broad range of events such as host membrane fission, *Salmonella*-containing vacuole maturation, and inflammation [29]. SopD is encoded outside SPI-1 and appears to be involved in membrane fission and macropinosome formation. SopE2, a guanine nucleotide exchange factor for Cdc42 of host cells, is important in membrane ruffling, bacterial entry and inflammation induction [26,30]. SptP is involved in inflammation and cytoskeleton manipulation of host cells [26]. SipA, SopB, SopD, and SopE2 have also been indicated to play roles in the late stage of *Salmonella* infection in mice [31]. Expression of avrA in *Salmonella* has been shown to increase bacterial invasion in the jejunum and colon and translocation in the spleen, liver, and gallbladder in chronically *Salmonella*-infected mice [32]. In the present study, the expression of all of these seven SPI-1 effectors was significantly down-regulated by both single and mixed LAB isolates. Compared with the corresponding high dose of LAB groups, the down-regulation was more evident at the low dose of LAB treatments except for the expression of *sopE2* by LB1 treatment only (Table 7).

Modulation of virulence gene expression in bacterial pathogens can be a mechanism underlying probiotic effects to control the infection of enteric pathogens. Previous *in vitro* studies have shown that lactobacilli and *Bifidobacterium bifidum* were able to down-regulate gene expression of *hilA* and *hilD* in *S.*Typhimurium and Enteritidis, respectively [13,14,15,16,17]. Similar *in vitro* observations have also been reported in *E. coli* O157:H7, in which *Lactobacillus acidophilus* repressed virulence gene expression and quorum sensing [33] and *Lactobacillus rhamnosus* reduced the expression of Shiga toxin genes (*stx*) [34]. The expression of eight Cag pathogenicity island genes was also down-regulated by *Lactobacillus salivarius* in a gastric epithelial cell line model [35]. *Salmonella* virulence gene expression can also be modulated by lactic acid and short-chain fatty acids (SCFAs). Lactate, propionate, and butyrate have been shown *in vitro* to down-regulate the expression of *Salmonella* virulence genes, such as *hilA*, *hilD*, *invF*, and *sipC* [36,37,38,39,40,41]. Acetate and formate were, however, reported to induce the *in vitro* expression of SPI-1 genes [36,42]. Some of our selected LAB isolates were able to produce lactic acid and SCFAs during fermentation (data not shown). It is unclear at present if the suppression of SPI-1 virulence gene expression of *Salmonella* in the chicken cecum described in the present study was caused by lactic acid and SCFAs produced by the isolates.

There is a lack of *in vivo* evidence on the reduction of *Salmonella* virulence gene expression by probiotics for control of the pathogen. The present study has generated a new piece of evidence demonstrating that the expression of intestinal infection-associated genes in *S.* Typhimurium can be down-regulated by selected LAB isolates when the pathogen was in the chicken cecum. This phenomenon coincided with an attenuation of *Salmonella* infection in the chicken spleen and liver. The evidence is further supported by our *in vitro* data, which revealed that co-culturing of ECF of *L.zeae* LB1 cultures with *Salmonella* resulted in a significant reduction in the expression of genes associated with *Salmonella* intestinal infection. These observations can thus lead to a hypothesis that metabolites from the LAB cultures are, at least in part, responsible for regulating the virulence gene expression in *Salmonella*, influencing *Salmonella* intestinal infection of chickens. Identification of the functional metabolites from LAB cultures would be the initial step to address the hypothesis, which can be facilitated by using *in vitro* assays for the virulence gene expression. Furthermore, identification of a dose response to the functional metabolites in down-regulating the virulence gene expression in *Salmonella in vivo* and in attenuating the organ infection of chickens remains critical in supporting the hypothesis.

Materials and Methods

In vitro characterization of lactic acid-producing bacterial isolates

Fourteen LAB isolates, including eight (ALB2, ALB6, CL9, L3, LB1, LB2, LB4, and S64) reported previously [18] and six used in the present study (K16, K67, S8, S33, S66, and SG2), were evaluated in triplicate *in vitro* for their tolerance to low pH and high bile salt and antibiotic resistance. The stock culture of each strain, stored at −80°C in 20% glycerol, was plated onto DeMan, Rogosa, and Sharpe (MRS) plates. Individual colonies were selected and inoculated into MRS broth, cultured at 37°C under anaerobic conditions (85% N_2, 10% CO_2, and 5% H_2) for 18 h, and the species identity of the six isolates were determined by sequencing 16S rRNA genes and comparison with the GenBank database using BLAST.

The susceptibility of the isolates to penicillin G, ciprofloxacin, tetracycline, erythromycin, ampicillin, and gentamicin was evaluated on MRS agar with MIC (Minimum Inhibition Concentration) Evaluator Strips (Oxoid, Basingstoke, UK) through gradient diffusion tests. The inhibitory zones generated by chloramphenicol and lincomycin were determined on the MRS agar using Oxoid Antimicrobial Discs (Oxoid, Basingstoke, UK). The range of antibiotics concentrations used in the test is listed in Table 3.

Tolerance to low pH was evaluated by incubating bacterial cells in simulated gastric fluid [43] with the pH values of 2.0 and 5.6, for 2 h prior to anaerobic incubation in the MRS broth at 37°C for 20 h. Tolerance to bile salt was studied by incubating the isolates in the MRS containing various concentrations (0, 0.3, 0.6, 1.5, and 1.5%) of bile salt (Catalog Number LP005, Oxoid, Nepean, ON, Canada) under anaerobic condition at 37°C for 24 h. The optical density (OD) of a sample was measured at a wavelength of 600 nm. The selected range of bile salts was based on the physiological concentration in the small intestine [44].

The effect of the ECF from a LAB culture (*L. zeae* LB1) on *in vitro* expression of *Salmonella* SPI-1 virulence genes was examined. To prepare the ECF, *L. zeae* LB1 was grown in MRS broth anaerobically at 37°C for 16 h and the ECF was recovered from the cultures (n = 3) by centrifugation (6,000 g, 10 min). After neutralized to pH 6.8–7.0 with NaOH (1 N), the ECF was sterilized through membrane filtration (0.2 μm pore size) and then mixed with the Luria–Bertani (LB) broth (1:1 ratio) that had double concentrations of each medium component. The ECF-LB mixtures were inoculated (1%) with *S.* Typhimurium P193, a nalidixic acid-resistant strain, and incubated at 37°C for 14 h prior to the treatment of *Salmonella* cells with RNAlater (Life Technologies Inc., Burlington, Canada).

Animal experiments

Newly hatched commercial female broiler chicks were obtained from Stratford Chick Hatchery (Stratford, ON, Canada). Birds were housed in floor pens containing wood shavings at the Isolation Unit of Ontario Veterinary College (University of Guelph, ON, Canada). Chickens had free access to water and a commercial starter diet without supplementation of antibiotics. The research protocol was approved by the University of Guelph Animal Care Committee and the guidelines of the Canadian Council for Animal Care were followed.

S. Typhimurium P193 was used to infect chickens according to the procedure described previously [45]. The procedure was

originally introduced by Mead et al. (1989) [46] for assessment of probiotic efficacy. *Salmonella* was cultured in LB broth at 37°C for 16 h, followed by dilution with sterilized phosphate-buffered saline (PBS, pH 7.2) to a final cell count of 4×10^4 CFU per ml for chicken infection experiments. To prepare LAB inoculum cultures, each isolate was propagated twice in MRS broth and then diluted to the desired concentrations with sterilized PBS. The desired concentrations were confirmed by plating on MRS plates after a series dilution.

In Trial 1, 72 one-day-old broilers were randomly assigned to 6 treatments: 1) negative control (no LAB + no *Salmonella*), 2) positive control (no LAB + *Salmonella* 1×10^4 CFU/chick), 3) LB1 (isolate LB1 1×10^6 CFU/chick + *Salmonella* 1×10^4 CFU/chick), 4) S8 (isolate S8 1×10^6 CFU/chick + *Salmonella* 1×10^4 CFU/chick), 5) S33 (isolate S33 1×10^6 CFU/chick + *Salmonella* 1×10^4 CFU/chick), 6) S64 (isolate S64 1×10^6 CFU/chick + *Salmonella* 1×10^4 CFU/chick). Each treatment had 12 chickens in one pen. Two rooms were used, one room for the negative control birds and another room for the remaining treatments. Chickens were orally gavaged with 0.5 ml of LAB or PBS (negative control) at 1 day of age and 0.25 ml of *S.* Typhimurium P193 at 2 days of age according to the experimental design. Four days after the challenge, all birds were humanely sacrificed by carbon dioxide. Cecal digesta samples were collected aseptically for *Salmonella* counts. Livers and spleens were collected from the positive control group to examine for possible *Salmonella* infection.

To follow up our observation on *Salmonella* infection to chicken spleens and livers in Trial 1, Trials 2 and 3 were designed to investigate the effect of *Lactobacillus* isolates on protecting broilers from organ infection by *Salmonella*, including the influence of the isolates on virulence gene expression of *Salmonella* in the cecum. There were six treatments in each trial: 1) negative control (no LAB + no *Salmonella*), 2) positive control (no LAB + *Salmonella* 1×10^4 CFU/chick), 3) low dose LB1 (isolate LB1 1×10^6 CFU/chick + *Salmonella* 1×10^4 CFU/chick), 4) high dose LB1 (isolate LB1 1×10^7 CFU/chick + *Salmonella* 1×10^4 CFU/chick), 5) low dose mixed isolates (isolates LB1, S8, and S64 1×10^6 CFU/strain/chick + *Salmonella* 1×10^4 CFU/chick), and 6) high dose mixed isolates (isolates LB1, S8, and S64 1×10^7 CFU/strain/chick + *Salmonella* 1×10^4 CFU/chick). Each treatment had 14 and 28 chickens in Trials 2 and 3, respectively. The maintenance of chickens, gavage of *Lactobacillus* cultures, and challenge of *Salmonella* were similar to those in Trial 1. Cecal digesta, livers and spleens were sampled aseptically on days 3 and 4 post-infection (PI) for *Salmonella* analysis in Trials 2 and 3. In addition, cecal digesta samples were aseptically collected on days 1 and 3 PI in Trial 3 and kept immediately in RNAlater (Life Technologies Inc., Burlington, Canada) for analysis of virulence gene expression of *Salmonella*.

Detection of cecal colonization and organ infection of *Salmonella*

On the same day of sample collection, cecal digesta samples were processed by a series 1:10 dilution with sterile solution of 0.1% peptone and 0.1% Tween 80 in water and plating on Brilliant Green Sulfa Agar (BGS; BD, Sparks, MD, USA) supplemented with 200 μg/ml nalidixic acid to facilitate selection of the antibiotic-resistant challenge organism. *Salmonella* colonies are pink to red on this selective media. The plating was accomplished by an Eddy Jet Spiral Plater (Neu-tec Group, Farmingdale, NY, USA). For detecting organ infection of chickens, about 0.1 g of spleen or liver samples were homogenized with PowerLyzer (MO BIO Laboratories, Carlsbad, CA,USA) by bead beating at homogenization level 2500 for 30 sec in a tube containing 1 ml of 0.1% peptone water and 0.5 ml of beads and incubated for 16 h at 37°C in 4 ml of Selenite Brilliant Green Sulfa Enrichment (Difco) broth (Sparks, MD, USA), followed by plating onto BGS supplemented with 200 μg/ml nalidixic acid to examine the presence or absence of *Salmonella* colonies. The recovery of *Salmonella* is reported as the ratio of the number of *Salmonella* positive samples to the number of total samples tested. The sensitivity of sample preparation (including both digesta and organ samples) and plating was assessed by including a known amount of *Salmonella* cells in the samples. The detection limit of our sample preparation and plating system was determined to be 100 CFU/g sample if the enrichment procedure was not applied.

Extraction of bacterial total RNA and reverse transcription/quantitative PCR (RT-qPCR)

Bacterial total RNA was extracted using the Ambion RiboPure-Bacteria Kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, samples of cecal digesta or *Salmonella* pure cultures that had been treated in RNAlater were washed with 2 volumes of PBS. After centrifugation (1500 x g) at 4°C for 10 min, the pellet was transferred to a new tube containing 0.5 ml beads and 0.8 ml RNAwiz provided with the Ambion RiboPure-Bacteria Kit and subjected to bead beating with PowerLyzer (MO BIO Laboratories, Carlsbad, CA, USA) for 2 min each at homogenization level 2500, repeated once. The tubes were chilled on ice for 2 min between bead beatings. Total bacterial RNA was then extracted, washed, and eluted following the manual of the kit. The RNA quantity was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

To remove genomic DNA contamination, total bacterial RNA samples were treated with DNaseI from the Ambion TURBO DNA-free kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Two micrograms of total RNA was then used for synthesis of cDNA with 100 ng of random hexamers and SuperScript II reverse transcriptase from the Invitrogen SuperScript II first-strand cDNA synthesis kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions.

The qPCR assays were performed using a Stratagene MX3005 thermal cycler (Stratagene, La Jolla, CA, USA) with the following thermal cycle profile: 95°C for 3 min for initial activation of the DNA polymerase; 40 cycles of 95°C for 15 sec (denaturation), specific temperature listed in Table 8 for 30 sec (annealing) and 72°C for 30 sec (extension). A thermal melt curve was generated by heating at 95°C for 1 min, 55°C for 30 sec, and ramping back to 95°C in 0.5°C increments. The 25 μl reaction mixture contained 1.0 μl of 3-fold diluted sample cDNA, 12.5 μl of 2× iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and forward and reverse primers (Table 8). The expression levels of virulence genes were normalized using 16S rDNA as an internal housekeeping control, and fold change of target genes was calculated by the delta-delta-Ct method [47]. ΔCt represents the difference between the Ct value with the primers to a target gene and the Ct value to the housekeeping gene (16S rRNA gene). ΔΔCt represents the difference in ΔCt values between the treatment and control groups. The values derived from $2^{-\Delta\Delta Ct}$ represent fold changes of samples in abundance relative to the reference samples. The reference samples had the $2^{-\Delta\Delta Ct}$ value of 1. The samples from the positive control chicken group in the chicken trial and the samples not subjected to the ECF treatment in the *in vitro* experiment served as the reference samples, respectively.

Table 8. PCR primers.

Gene	Primers (5′-3′)	Amplicon size (bp)	Primer final concentration (nM)	Annealing temperature (°C)	Reference
16S rRNA	Forward: CAGAAGAAGCACCGGCTAACTC	87	150	60	[48]
	Reverse: GCGCTTTACGCCCAGTAATT				
hilA	Forward: CATGGCTGGTCAGTTGGAG	150	300	62	[49]
	Reverse: CGTAATTCATCGCCTAAACG				
hilC	Forward: GGACTTGTTGCCAGGGATG	241	300	62	[49]
	Reverse: TGACCATTTGCGGGTGAG				
hilD	Forward: ACTCGAGATACCGACGCAAC	129	300	62	[49]
	Reverse: CTTCTGGCAGGAAAGTCAGG				
sopB	Forward: AACCGTTCTGGGTAAACAAGAC	77	500	56	[50]
	Reverse: GGTCCGCTTTAACTTTGGCTAAC				
sopD	Forward: CTTTAAGCTTCGGTAATCATCAAAA	306	500	50	[51]
	Reverse: AAGCGTCCATCTTGATAGTAAACAG				
sopE2	Forward: GCCTGCATCAACAAACAGACA	72	500	60	[52]
	Reverse: ATACCGCCCTACCCTCAGAAG				
sipA	Forward: GGCTTGCGTGCGGAAATA	69	500	60	[53]
	Reverse: ATCGCTACATTGCGCTTTCA				
sipC	Forward: CTGTGGCTTTCAGTGGTCAG	150	500	60	[49]
	Reverse: TGCGTTGTCCGGTAGTATTTC				
avrA	Forward: GGAAACCGATCTCGAAATGA	241	500	57	[54]
	Reverse: TGCTGGTTCGAACAAAATCA				
sptP	Forward: ATGCTCGTGCCTGGTGGTGTTA	236	150	60	This study
	Reverse: ACGGTAACGGCTGGTGATCT				

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Statistical analysis

Values were expressed as mean ± standard error. Tolerance to low pH and high bile salt and gene expression data were analyzed by the SAS GLM procedure and Tukey multiple comparisons. *Salmonella* counts in cecal contents were log transformed and subjected to analysis of variance and Dunnett's test for multiple comparisons. Logistic regression was used for analysis of data on *Salmonella* infection in the liver and spleen. When data from animals in Trials 2 and 3 were pooled, mixed model was employed with trial as a fixed effect.

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Author Contributions

Conceived the research: JG SS PS. Designed the experiments: XY HY JB JG SS. Performed the experiments: XY JB HY QW FY YZ. Analyzed the data: XY HY. Contributed reagents/materials/analysis tools: JG SS. Wrote the paper: XY JG.

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