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In vitro and *in vivo* activity of *Lactobacillus sakei* L14 strain against *Campylobacter jejuni* DC3 strain

John Roybert P. Catacutan, Ma. Socorro Edden P. Subejano, Gil M. Penuliar✉

Institute of Biology, National Science Complex, College of Science
University of the Philippines Diliman, Quezon City 1101, Philippines
gmpenuliar@up.edu.ph

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Abstract

Introduction: Domestic poultry is a natural reservoir of *Campylobacter*, the host–pathogen interaction being predominantly asymptomatic. This study investigated whether chickens remain asymptomatic partly because of lactic acid bacteria (LAB). **Material and Methods:** *Campylobacter* spp. and LAB were isolated from the gut of poultry chickens using enrichment and screening assays and were identified *via* rDNA sequencing. The *C. jejuni* DC3 isolate was grown in different cell-free supernatants (CFS) generated from a priority LAB isolate. An *in vivo* challenge involving the *C. jejuni* and LAB isolates using a chicken model was performed to confirm the *in vitro* findings. **Results:** Twelve presumptive LAB isolates had anti-*C. jejuni* activity based on cross-streak and agar plug assays, with *Lactobacillus sakei* L14 isolate exhibiting the highest activity. Inhibition by *L. sakei* L14 CFS of the growth of *C. jejuni* occurred in a dose-dependent manner. *Campylobacter jejuni* DC3 inhibition was most evident in CFS harvested at 72 h and produced by co-culture with the pathogen. Neutralisation of the CFS abrogated the observed inhibition. Co-infection with *C. jejuni* DC3 and *L. sakei* L14 *in vivo*, however, failed to inhibit *C. jejuni* colonisation in chickens. **Conclusion:** The results suggest that the anti-*C. jejuni* effect of *L. sakei* L14 in chickens may be due to mechanisms other than direct inhibition of growth.

Keywords: lactic acid bacteria, *Lactobacillus sakei*, *Campylobacter jejuni*, chickens, growth inhibition.

Introduction

Campylobacter is one of the most important enteropathogens that cause diarrhoeal diseases. The diseases with this aetiology, referred to as campylobacteriosis, are commonly manifested as gastroenteritis accompanied by mild to severe diarrhoea. The incidence of human campylobacteriosis mainly caused by *C. jejuni* and *C. coli* has been increasing worldwide. In some parts of the world, it exceeds those of salmonellosis and shigellosis. The main mode of transmission is *via* the ingestion of contaminated food, primarily chicken (3), and this species is considered a natural reservoir of *Campylobacter*, as are turkeys and ducks. Birds are infected via the faecal–oral route and are primarily colonised in the gut. The prevalence of *Campylobacter* in poultry has been observed to vary depending on geographical location, season, and production system (3, 21). The host–pathogen interaction between chicken and *Campylobacter* is predominantly commensal and asymptomatic; however, studies have shown that *Campylobacter* is capable of invading gut cells and entering blood vessels, causing extraintestinal infection of other organs such as the liver and spleen. Evidence suggests that chickens mount an immune response to *Campylobacter* and other commensal bacteria (21). In addition to *Campylobacter*, lactic acid bacteria (LAB) are also present in the gastrointestinal tract of birds and these bacteria constitute a Gram-positive, non-spore-forming group that produce lactic acid as the major end-product of carbohydrate fermentation. As examples of LAB that have been isolated from chickens, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus* have been shown to produce antibacterial substances such as organic acids, hydrogen peroxide, carbon dioxide, and bacteriocins (13, 19).

The objective of this study is to determine whether the natural resistance of chickens to *Campylobacter* is mediated by LAB. Specifically, this work aimed to isolate *C. jejuni* and LAB from the gastrointestinal tracts of healthy poultry chickens; determine the interaction between the isolated *C. jejuni* and LAB *via* cell-to-cell contact; determine if the cell-free supernatant of LAB is

capable of inhibiting *C. jejuni*, and determine the effect of *C. jejuni* co-infection with LAB in an *in vivo* chicken model. This study determined the role of LAB in *C. jejuni* colonisation in chickens and may contribute to the current knowledge as to why chickens remain asymptomatic despite being colonised by *Campylobacter*.

Material and Methods

Sample collection. A 4-week-old broiler chicken was obtained from a wet market in the Philippines. The chicken was humanely euthanised *via* cervical dislocation following the recommendations of the Handbook of Laboratory Animal Management and Welfare (29). The small intestine, large intestine, and cecum were dissected and stored in an ice-cold airtight container, which was transported to the laboratory within 4 h of collection. The samples were homogenised in 10^{-1} volumes of 0.9% sterile saline. The resulting mixture was weighed and equally divided for the isolation of *Campylobacter* and endogenous LAB. For exogenous LAB, five poultry chicken intestinal tracts were procured from wet markets in Metro Manila. The gut samples from all five birds were pooled and homogenised in 10^{-1} volumes of 0.9% sterile saline.

Isolation and biochemical tests. The enrichment protocol for *Campylobacter* using Bolton broth as specified in the ISO 10272-1:2017 standard (11) was followed with modifications. Briefly, 25 mL of the homogenised sample was enriched in 100 mL of Bolton selective enrichment broth supplemented with cefoperazone (20 mg/L), vancomycin (20 mg/L), trimethoprim (20 mg/L), cycloheximide (50 mg/L), piperacillin-tazobactam (8 mg/1 mg/L), and 5% lysed horse blood. The culture was pre-enriched at 37°C for 4 h under microaerobic conditions and incubated at 42°C for 48 h under the same conditions. The enrichment was streaked onto chromogenic Mueller–Hinton agar (MHA) plates supplemented with cefoperazone (32 mg/L), amphotericin B (10 mg/L), piperacillin-tazobactam (8 mg/1 mg/L), and 5% lysed horse blood and the plates were incubated at 42°C for 48 h under microaerobic conditions. Colonies that were dark matte purple coloured were selected for purification. Presumptive *Campylobacter* spp. isolates were subcultured on MHA plates with 5% lysed horse blood and incubated at 42°C for 48 h under microaerobic conditions for DNA extraction and PCR amplification. For LAB isolation, 10 mL of each homogenised sample was serially diluted to 10^{-6} in 0.9% sterile saline solution. A 100 µL volume of each of the last three dilutions (10^{-4}, 10^{-5}, and 10^{-6}) was pipetted onto de Man, Rogosa, and Sharpe (MRS) agar plates supplemented with 0.5% $CaCO_3$. The plates were incubated at 37°C for 48–72 h under microaerobic conditions. Colonies with zones of clearing were purified on MRS agar plates, which were incubated as previously described. Gram staining and a catalase test were performed based on the protocols of Smith and Hussey (25) and Reiner (20), respectively, and a hippurate hydrolysis test was conducted as described by Morris *et al*. (18).

Screening for antimicrobial activity. Primary screening was performed *via* a cross-streak assay. Lactic acid bacteria isolates were seeded on MHA plates with 5% lysed horse blood until one-third of the surface was covered. The plates were then incubated at 37°C for 48 h under microaerobic conditions. On the free surface of the same MHA plates, the *C. jejuni* isolate and the *C. jejuni* ATCC 33560 quality control strain (Microbiologics, St. Cloud, MN, USA) were aseptically streaked horizontally towards the LAB growth. Streaking of the test pathogens was performed in duplicate. The plates were incubated at 42°C for 48 h under microaerobic conditions, after which the LAB isolates that exhibited inhibitory activity against the *C. jejuni* isolate and the control organism *C. jejuni* ATCC 33560 were selected for secondary screening.

Secondary screening was performed using the agar plug diffusion method. Lawns of LAB were prepared on MHA plates with 5% lysed horse blood, using cultures adjusted to 0.5 McFarland. A sterile swab previously dipped once into tubes containing the adjusted inoculum was streaked three times on the MHA plates to cover the entire surface of the medium. The plates were then incubated at 37°C for 48 h under microaerobic conditions. Bacterial lawns of the *C. jejuni* isolate were prepared as previously described using cultures adjusted to 0.5 McFarland and streaked onto MHA plates with 5% lysed horse blood. Five-millimetre agar plugs were bored from LAB-seeded plates and were aseptically transferred onto the *C. jejuni* isolate lawns. The plates were incubated at 42°C for 48 h under microaerobic conditions, after which the areas of inhibition were measured using a Vernier caliper.

Molecular identification. Genomic DNA of the presumptive *Campylobacter* isolate and the priority LAB isolate were extracted using a G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, South Korea), following the manufacturer's recommendations. A 20 µL PCR reaction mixture composed of 10 µL of GoTaq Master Mix (Promega, Wisconsin, USA) (with 50 units/mL of Taq DNA polymerase in a proprietary reaction buffer (pH 8.5), 400 µM each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and 3 mM of $MgCl_2$), 1.2 µL of each primer (0.6 µM), 6.6 µL of nuclease-free water, and 1 µL of template DNA was prepared. Amplifications were carried out in a Labnet MultiGene Gradient PCR thermal cycler (Labnet International, Edison, NJ, USA) using *Campylobacter* spp.- and *C. jejuni*-specific primers (Table 1) and following the optimised PCR conditions described by Subejano and Penuliar (26). The genomic DNA of the *C. jejuni* isolate and the priority LAB isolate were sent to Macrogen Inc.

(Seoul, South Korea) for DNA sequencing. The consensus sequences of the forward and reverse sequences were obtained using Bioedit Sequence Alignment Editor v. 7.0.5 (6) and used to identify the isolates through cross-referencing with the nucleotide sequences deposited in the National Center for Biotechnology Information Nucleotide BLAST database.

Preparation and collection of cell-free supernatant. The growth of *C. jejuni* DC3 isolate was determined in broth cultures with different preparations of cell-free supernatant (CFS). Two types of CFS were collected: CFS-MC from a monoculture of *L. sakei* L14 isolate and CFS-CC from a co-culture of *L. sakei* L14 and *C. jejuni* DC3. For CFS-MC, 1 mL of 1.0 McFarland-adjusted LAB culture was inoculated into 149 mL of yeast autolysate–peptone–tryptone–Tween 80–glucose (LAPTg) broth. For CFS-CC, 1 mL each of 1.0 McFarland-adjusted LAB and *C. jejuni* isolate were inoculated together into 148 mL of LAPTg broth. The preparations were incubated at 37°C under microaerobic conditions. Approximately 30 mL of the cultures were harvested after 24, 48, and 72 h of incubation by centrifugation at 12,000 rpm for 1 min. The supernatants were collected, passed through a 0.45 µm polyether sulfone syringe filter (Membrane Solutions, Shanghai, China), and pooled in 50 mL conical tubes.

***Campylobacter jejuni* in LAB CFS.** *Campylobacter jejuni* DC3 was cultured in different concentrations of CFS-MC and CFS-CC in separate scintillation vials for final volumes of 10 mL. A single concentration of *C. jejuni* DC3 (3.0×10^8 CFU/mL) was used in all treatments. One millilitre of the 1.0 McFarland-adjusted *C. jejuni* suspension was cultured in CFS-MC and CFS-CC, each with five different proportions of CFS (0%, 12.5%, 25%, 50%, and 100%) harvested after three different timepoints (24, 48, and 72 h). Uninoculated vials with only the culture medium were prepared to serve as sterility controls. Each treatment was performed in triplicate. The preparations were incubated at 42°C under microaerobic conditions. To measure the growth of *C. jejuni* DC3, spectrophotometry was performed after 48 h of incubation with the LAB CFS, and absorbance readings were used to plot growth curves using Microsoft Excel (Microsoft, Redmond, WA, USA).

***Campylobacter jejuni* in neutralised and heat-treated CFS.** CFS-MC and CFS-CC were neutralised using 1 N NaOH. Scharlau pH indicator strips (Scharlab, Barcelona, Spain) were used to monitor the changes in pH. A separate set of ten-millilitre volumes of CFS-MC at the proportions previously stated and an equivalent set with CFS-CC were heat-treated at 121°C and 15 psi for 15 min using a BKQ-B II vertical autoclave (BIOBASE, Shandong, China). *Campylobacter jejuni* DC3 was cultured in different concentrations of CFS in separate scintillation vials for final volumes of 10 mL. The CFS that were harvested after 72 h were used for each set-up. A single concentration of *C. jejuni* DC3 (3.0×10^8 CFU/mL) was used in all treatments. One millilitre of the 1.0 McFarland-adjusted *C. jejuni* suspension was cultured in both the CFS previously described, each with three different proportions of CFS (0%, 50%, and 100%) harvested after 72 h. As previously, uninoculated vials with only the culture medium were prepared to serve as sterility controls. Each treatment was performed in triplicate. In this operation also, the preparations were incubated at 42°C under microaerobic conditions. To measure the growth of *C. jejuni* DC3, spectrophotometry was performed after 48 h of incubation with the CFS and absorbance readings were used to plot growth curves using Microsoft Excel.

Statistical analysis. Significant differences in the absorbance readings obtained for each type of CFS, concentration of CFS, and incubation times used were determined using analysis of variance (ANOVA) ($\alpha = 0.05$) and post-hoc Tukey's Honestly Significant Difference (HSD) test.

***In vivo* challenge with *C. jejuni* and LAB.** Twenty 1-day-old female Cobb broiler chicks were procured and were housed in an animal facility outside the University of the Philippines. Four groups were established: Group 1 for no treatment, Group 2 for inoculation with *C. jejuni* DC3 only (control group), Group 3 for inoculation with *C. jejuni* DC3 and *L. sakei* L14 concurrently, and Group 4 for inoculation with *L. sakei* L14 only. Separate cages with five chicks each were prepared for each treatment. The chicks were acclimated for 7 days prior to any treatment. The temperature was maintained at 30–36°C and monitored daily and adequate internal air circulation was provided. Sterile commercial chick starter mash/feeds (GMP 100) and sterile drinking water were given *ad libitum* using designated containers. The chicks were given antibiotics on days 1–7 to deplete their existing gut microflora, following the protocol by Han *et al.* (7) with modifications. The antibiotic cocktail was composed of broad-spectrum antibiotics, specifically doxycycline (0.5 mg/mL), tiamulin (0.12 mg/mL), amoxicillin (0.125 mg/mL), tylosin (0.08mg/mL), ciprofloxacin (0.125 mg/mL), sulfadimethoxine (0.2 mg/mL), and trimethoprim (0.04 mg/mL), dissolved in sterile drinking water. Faeces were collected using sterile cotton swabs and transferred into 5 mL of Bolton broth with 5% lysed horse blood and 5 mL of MRS broth on day 8. The samples were transported to the lab where they were incubated at 42°C for *C. jejuni* and 37°C for LAB for 48 h under microaerobic conditions. The enrichments were processed for DNA extraction and were screened by PCR for *Campylobacter* spp. and *L. sakei*. Primer sequences for the LAB group and the target LAB were adapted from the literature (26) and are shown in Table 2, and PCR conditions were optimised in the laboratory.

Upon confirming the absence of the target bacteria, each group was treated with *C. jejuni* and *L. sakei* *via* ingestion of a standardised bacterial suspension every 2 days for 31 days. Approximately 25 mL of *C. jejuni* and LAB at 9×10^8 CFU/mL concentration were prepared separately in lactated Ringer's solution.

On each second day, 25 mL of the standardised bacterial suspension was administered to each group of chicks. Group 1 was neither infected with *C. jejuni* nor LAB. Group 2 was infected with *C. jejuni* only for 31 days between days 8 and 39. Group 3 was simultaneously infected with *C. jejuni* and LAB for 31 days between the same start and end points, and Group 4 was infected with LAB only with identical duration parameters. Faecal samples were collected and processed as previously described to confirm the presence of *C. jejuni* and LAB at 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 and 31 days post infection (dpi). At the end of the experiment, the chickens were humanely euthanised *via* cervical dislocation in order for their digestive tract tissue to be sampled to detect the presence of *C. jejuni* *via* PCR.

Results

Ten presumptive *Campylobacter* spp. were isolated, but only isolates DC1, DC2, DC3, and DC4 were initially identified as *C. jejuni* using species-specific primers. These four isolates were all Gram-negative and hippurate-positive. The third one, DC3, was chosen as the priority isolate due to its sustained viability. Sequencing of its 16S rDNA region identified DC3 as *C. jejuni* with 99.9% identity.

There was successful isolation and purification of 12 endogenous and 30 exogenous LAB. The exogenous LAB isolate L14 was chosen as the priority isolate based on its high activity against *C. jejuni* DC3 and *C. jejuni* ATCC 33560, in addition to its sustained viability. It was determined to be Gram-positive and catalase-negative. DNA sequencing of the16S rDNA region of L14 identified it as *Lactobacillus sakei*. The endogenous LAB isolates did not show any inhibitory activity against *C. jejuni* DC3 or *C. jejuni* ATCC 33560 (data not shown). For the exogenous LAB, 12 isolates inhibited the growth of *C. jejuni* DC3 and *C. jejuni* ATCC 33560 (Fig. 1). Only 10 isolates were subjected to secondary screening, however, because of loss of viability affecting two isolates. Six LAB isolates showed consistent activity against both *C. jejuni* DC3 and *C. jejuni* ATCC 33560, with *L. sakei* L14 exhibiting the most activity (Fig. 2).

Lactobacillus sakei L14 CFS generated from the co-culture with and without *C. jejuni* DC3 was able to inhibit *C. jejuni* DC3 growth in a dose-dependent manner (Figs 3 and 4). Co-culture–derived CFS harvested at 72 h was most efficient in inhibiting *C. jejuni* DC3 growth, starting at a 25% proportion of the suspension being supernatant. Neutralised *L. sakei* L14 CFS was unable to inhibit *C. jejuni* DC3 growth ($P > 0.05$), as shown in Fig. 5. On the other hand, heat-treated *L. sakei* L14 CFS harvested after 72 h was shown to inhibit *C. jejuni* DC3 growth to greater extents as its proportion increased, as seen in Fig. 6. Significantly different values between the 0% and 50% and the 0% and 100% proportions were determined using ANOVA ($\alpha = 0.05$) and post-hoc Tukey's HSD test.

PCR detection of *C. jejuni* DC3 from pooled faecal samples from each preparation shows that colonisation of *C. jejuni* occurred as early as 5 dpi (Table 3a). *Campylobacter jejuni* was only detected once in Group 2 (infected only with this bacterium) during the 31-day infection period, while in Group 3 (infected with this bacterium and LAB) it was detected, although not consistently, at several points during the observation period (Table 3a).

Lactobacillus sakei L14 was detected as early as 1 dpi in groups 3 and 4 (the former infected with bacteria of both genera). In Group 3, it was evident at 13, 19, and 21 dpi and in Group 4 from 19 to 25 dpi. However, it was undetectable in Group 3 and Group 4 at 23 dpi and 27, respectively (Table 3b).

Table 3c shows that LAB were consistently detected in all treatment groups before and during treatment. The results of PCR amplification of *C. jejuni* from individual gut samples were consistent with the results of assays to detect it in the pooled faecal samples, suggesting that *C. jejuni* DC3 was able to successfully colonise the chickens in the co-infection group (Table 4).

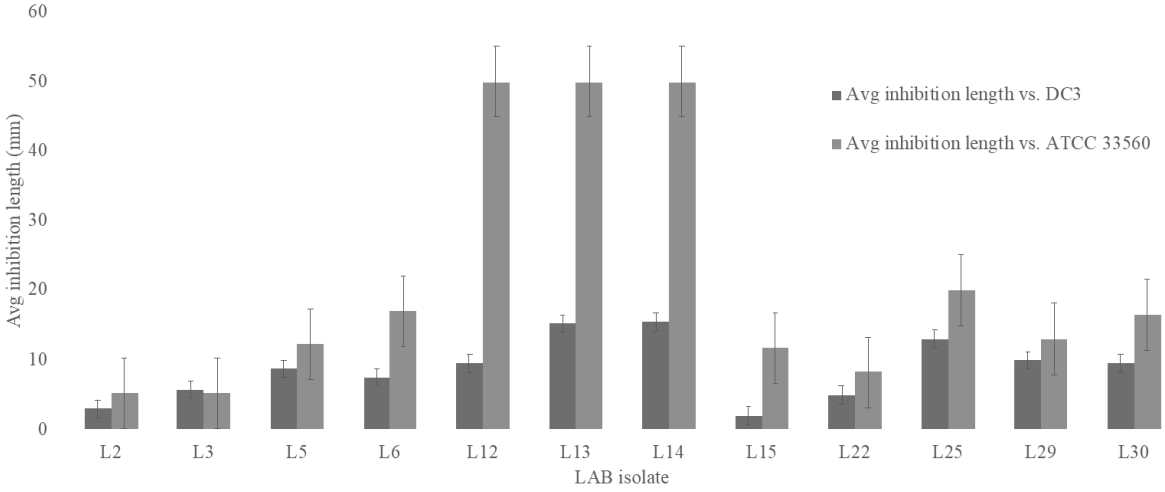

Fig. 1. Primary screening results of putative exogenous lactic acid bacteria with inhibitory activity against *Campylobacter jejuni* DC3 and *C. jejuni* ATCC 33560. Error bars represent standard error

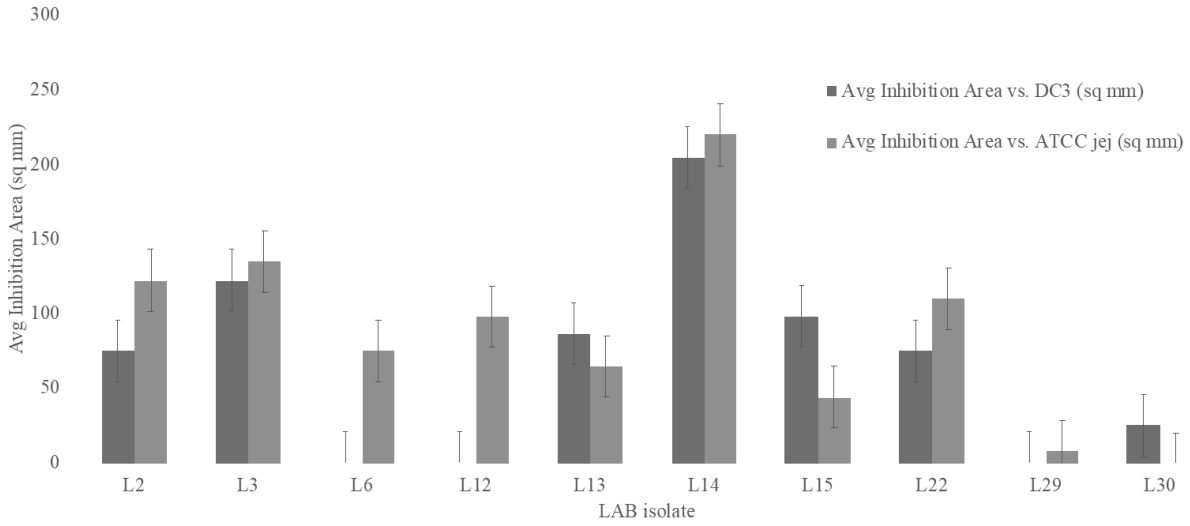

Fig. 2. Secondary screening results of putative exogenous lactic acid bacteria against *Campylobacter jejuni* DC3 and *C. jejuni* ATCC 33560. Error bars represent standard error

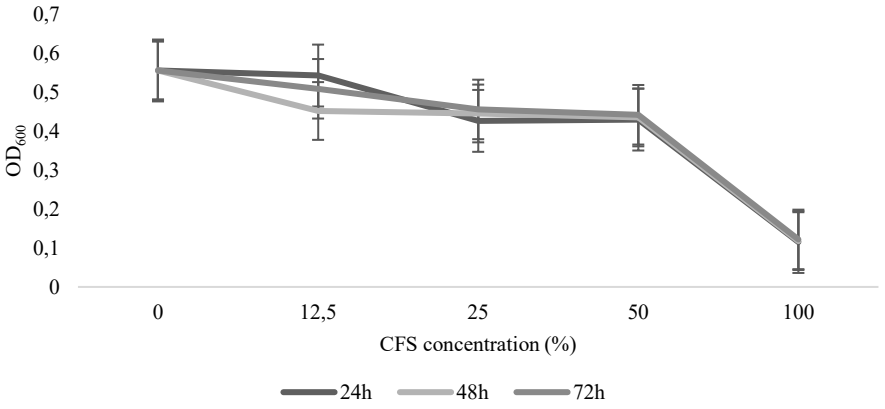

Fig. 3. Growth curve of *Campylobacter jejuni* DC3 grown in *Lactobacillus sakei* L14-only cell-free supernatant (CFS) from 3 different timepoints (24, 48, and 72 h) and 5 different proportions (0%, 12.5%, 25%, 50%, 100%), based on absorbance readings (optical density (OD) 600 nm). Error bars represent standard error

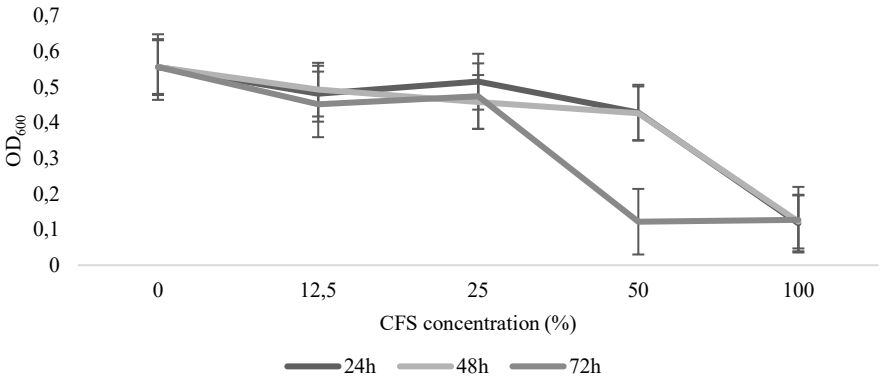

Fig. 4. Growth curve of *Campylobacter jejuni* DC3 grown in *Lactobacillus sakei* L14 co-cultured with *C. jejuni* DC3 cell-free supernatant (CFS) from 3 different timepoints (24, 48, and 72 h) and 5 different proportions (0%, 12.5%, 25%, 50%, 100%), based on absorbance readings (optical density (OD) 600 nm). Error bars represent standard error

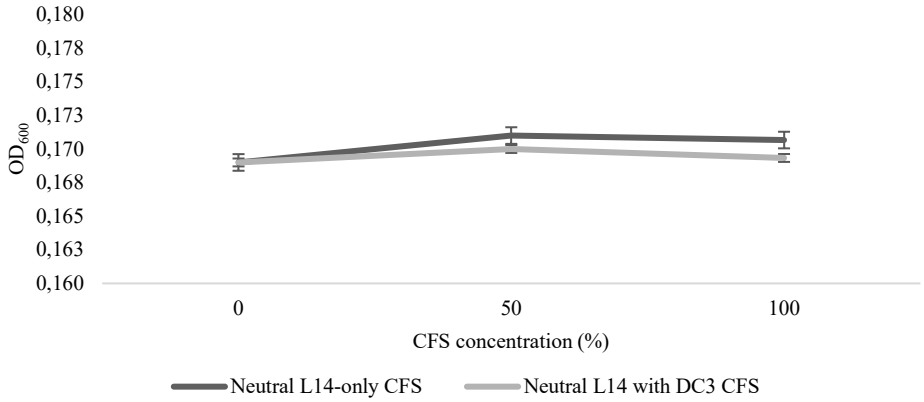

Fig. 5. Growth curve of *Campylobacter jejuni* DC3 in neutralised *Lactobacillus sakei* L14 only and neutralised *L. sakei* L14 with *C. jejuni* DC3 cell-free supernatant (CFS) from a single timepoint (72 h) and in 3 different proportions, based on absorbance readings (optical density (OD) 600 nm). Error bars represent standard error. Analysis of variance (α = 0.05) indicated no significant difference across all treatments for both types of CFS

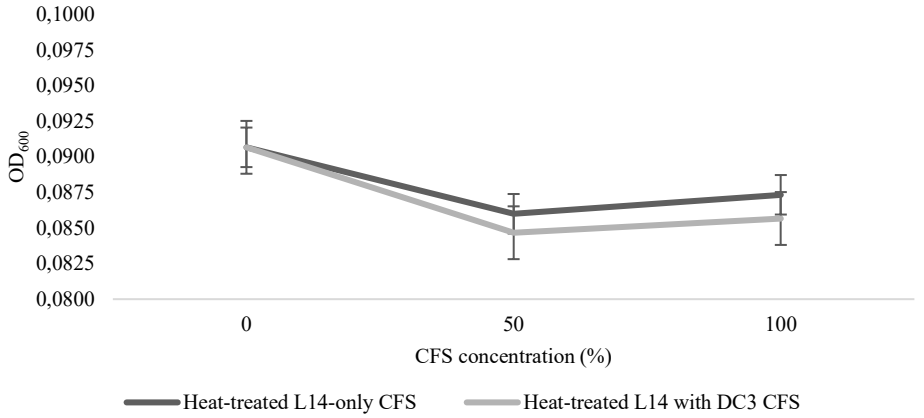

Fig. 6. Growth curve of *Campylobacter jejuni* DC3 in heat-treated *Lactobacillus sakei* L14 only and *L. sakei* L14 with *C. jejuni* DC3 cell-free supernatant (CFS) from a single timepoint (72 h) and in 3 different proportions, based on absorbance readings (optical density (OD) 600 nm). Error bars represent standard error. Analysis of variance (α = 0.05) and post-hoc Tukey's Honestly Significant Difference test indicated significant differences between 0 and 50% and 0 and 100% proportions for both types of CFS

Table 1. Primers used in this study

Primer	Sequence (5′-3′)	PCR product (bp)	Target organism	Reference
MD16S1-F	ATCTAATGGCTTAACCATTAAAC	857	*Campylobacter* spp.	(10)
MD16S2-R	GGACGGTAACTAGTTTAGTATT			
MDmapA1-F	CTATTTTATTTTTGAGTGCTTGTG	589	*C. jejuni*	
MDmapA2-R	GCTTTATTTGCCATTTGTTTTATTA			
L15f	GCTCAGGAYGAACGCYGG	750	lactic acid bacteria	(9)
L687r	CACCGCTACACATGRADTTC			
lsF	GAGCTTGCTCCTCATTGATAA	434	*Lactobacillus sakei*	(17)
lsR	TTGGATACCGTCACTACCTG			

F and f – forward; R and r – reverse

Table 2. Optimised PCR conditions used in this study adapted from Subejano & Penuliar (26)

PCR step	MD16S1-F / MD16S2-R	MDmapA1-F / MDmapA2-R	L15f / L687r	lsF / lsR	Number of cycles
Initial denaturation	95°C, 2 min	95°C, 2 min	95°C, 2 min	95°C, 2 min	1
Denaturation	95°C, 1 min	95°C, 1 min	95°C, 1 min	95°C, 1 min	35
Annealing	49°C, 1 min	53°C, 1 min	60°C, 1 min	60°C, 1 min	
Extension	72°C, 1 min	72°C, 1 min	72°C, 1 min	72°C, 1 min	
Final extension	72°C, 5 min	72°C, 5 min	72°C, 5 min	72°C, 5 min	1

F and f – forward; R and r – reverse

Table 3a. *Campylobacter* spp. detection results from pooled faecal samples

Group	Days post infection																
	pre	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
3	-	-	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group 1 – Untreated; Group 2 – *Campylobacter jejuni* DC3 only; Group 3 – *C. jejuni* DC3 and *Lactobacillus sakei* L14 co-infection; Group 4 – *L. sakei* L14 only

Table 3b. *Lactobacillus sakei* L14 PCR detection results from pooled faecal samples

Group	Days post infection																
	pre	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-
4	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-

Group 1 – Untreated; Group 2 – *Campylobacter jejuni* DC3 only; Group 3 – *C. jejuni* DC3 and *L. sakei* L14 co-infection; Group 4 – *L. sakei* L14 only

Table 3c. Lactic acid bacteria PCR detection results from pooled faecal samples

Group	Days post infection																
	pre	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Group 1 – Untreated; Group 2 – *Campylobacter jejuni* DC3 only; Group 3 – *C. jejuni* DC3 and *Lactobacillus sakei* L14 co-infection; Group 4 – *L. sakei* L14 only

Table 4. *Campylobacter* spp. PCR detection results from individual gut samples at 33 days post infection

Group	Sample			
	A	B	C	D
1	-	-	-	n/a
2	-	-	-	n/a
3	+	+	+	+
4	-	-	-	-

Discussion

Lactic acid bacteria are a diverse group of microaerophilic organisms that can be isolated from many sources, especially fermented foods and animal intestines. Aside from their major role in the food technology industry because of their antimicrobial activity, probiotic LAB have also been considered as an alternative therapy in alleviating discomfort caused by gastrointestinal infections (22). Their antibacterial property is based on the production of lactic acid and secretion of growth-inhibiting substances such as hydrogen peroxide, diacetyl, and bacteriocins (15). One of these bacteria is *Lactobacillus sakei*, which is naturally found on raw and fermented meats (15, 30). Despite being widely associated with animal muscle tissue and therefore, with food, it has also been isolated from chicken intestines (1, 24), a finding that was confirmed in this study. While there have been numerous studies on *L. sakei* demonstrating its use as a protective culture for meat products (14, 30), there have also been reports on its probiotic potential, especially in the poultry industry (22, 24).

Lactobacillus sakei and other exogenous LAB in this study exhibited anti-*C. jejuni* activity *in vitro* through direct cell-to-cell contact in screening assays. In a similar study, Jones *et al*. (13) were also able to report the same activity by two strains of *L. sakei* from meat products. It is interesting to note that none of the endogenous LAB or those isolated from the same chicken intestine as *C. jejuni* DC3 inhibited the growth of the *Campylobacter* isolate *via* direct cell-to-cell contact. It is possible that these LAB isolates are species other than *Lactobacillus* that do not produce secretory products capable of inhibiting the growth of *Campylobacter*. It should also be noted that only a small number of endogenous LAB were isolated (n = 12) and it is likely that other isolates with potential anti-*C. jejuni* activity were present but were not selected during isolation. It is also possible that these LAB isolates exert their antagonistic effects through indirect mechanisms of action. Studies have demonstrated how *C. jejuni* inhibition is LAB strain- and species-specific (16, 22).

In this study, *C. jejuni* DC3 growth was inhibited by CFS-MC and CFS-CC, but significant pathogen

growth inhibition was observed only by CFS-CC generated after 72 h of incubation. Co-incubation of LAB with its target pathogen has been shown to be effective in inhibiting growth. Drago *et al.* (5) reported the same findings in *L. paracasei* that was able to inhibit the growth of *Escherichia coli* and *Salmonella enteritidis in vitro*, either in the simultaneous inoculation setup or when the pathogens were added after *L. paracasei* was first grown overnight. The inhibitory substances produced by LAB are secreted when the bacteria are grown in an appropriate medium. In testing the inhibitory activity of the CFS in this work, LAPTg broth was the culture medium of choice because it is the conventional medium used in LAB cultivation and has been reported to be the optimal medium for bacteriocin production. It also has lower amounts of potentially interfering peptides compared to MRS (4). There are studies that have attributed the antimicrobial activity of LAB CFS against Gram-negative pathogens to lactic acid accumulation and the concomitant lowering of pH (28) and which have posited that neutralisation will eliminate the antagonistic effect of LAB (16, 28), as observed in this study. The antibacterial activity of lactic acid and other organic acids is said to be due to their ability to penetrate the cytoplasmic membrane in their undissociated forms. This results in a reduction of the intracellular environment's pH and a subsequent disruption of the proton motive force. In Gram-negative bacteria, lactic acid has the ability to eliminate the lipopolysaccharide layer, which confers the permeability barrier property of the outer membrane. This consequently disrupts the outer membrane, which may enhance the action of other antimicrobial substances (2).

The potential of *L. sakei* L14 to produce bacteriocins that can inhibit *C. jejuni* was preliminarily explored with the use of LAPTg broth as growth medium. Bacteriocins exhibit species-specific or broad-spectrum antimicrobial activity and may be heat stable or heat labile. Examination of the bacteriocinogenic potential of *L. sakei* L14 after heat treatment revealed that heat-treated CFS-MC and CFS-CC retained their anti-*C. jejuni* activity, but did not show any further pathogen growth inhibition, even with increased CFS concentration.

Despite the evident *in vitro* activity of *L. sakei* L14 against *C. jejuni* DC3, the results of the chicken model experiment demonstrate how the LAB isolate failed to inhibit the pathogen *in vivo*. Although many LAB have been examined for their anti-*Campylobacter* activity *in vitro*, only a few studies have confirmed this effect *in vivo*. The LAB-associated *Bifidobacterium longum* PCB 133 actinobacterium showed anti-*C. jejuni* activity *in vitro* and *in vivo* (23). Another study reported that *L. crispatus* JCM 5810, *L. acidophilus* NCFM, *L. gallinarum* ATCC 33199, and *L. helveticus* CNRZ32 were able to reduce the level of *C. jejuni* colonisation in broiler chicks (19). There are different mechanisms by which LAB can reduce *Campylobacter* colonisation in the gut. Aside from the production of organic acids and bacteriocins, LAB may also compete with pathogens for nutrients or strengthen the tight junctions in the intestinal epithelium to inhibit pathogen invasion. Lactic acid bacteria can also colonise the intestinal epithelium so that pathogenic bacteria cannot adhere and invade epithelial cells or may directly bind to the pathogen. Immune system modulation by LAB is also a possible mechanism that may facilitate the host's mounting an effective immune response against pathogens (22).

The *in vivo* experiment was more complex and included host factors that could have influenced the activity of *L. sakei* L14, such as avian gut morphology and health, the microbial communities present, and the avian immune system. Taking into consideration these host factors and the different mechanisms by which LAB can deliver beneficial effects to its host, it is likely that *L. sakei* L14 confers its protective effect through mechanisms other than the production of organic acids and antimicrobial substances. Sim *et al*. (24) reported that in the murine model, heat-killed cells of two *L. sakei* strains were able to induce high levels of immune cell proliferation as well as stimulate the production of interleukin (IL) 6 and tumour necrosis factor alpha (TNF-α). Hong *et al*. (8) were also able to show the immune-inducing potential of *L. sakei* in the murine model when it exhibited the ability to stimulate IL-12, interferon gamma, and TNF-α production. Based on these previous findings, it is possible that *L. sakei* L14 may have also stimulated the avian immune system, in addition to stimulating organic acid production and probably the secretion of antimicrobial peptides.

Although *L. sakei* strains have been isolated from human faeces, they are most often isolated from food products such as fermented cabbage and meat. Because of its presence in food, *L. sakei* is mostly considered a diet-associated LAB, which implies that the gastrointestinal tract may not be its natural environment (30). This may help explain why *L. sakei* L14 did not inhibit the pathogen's growth *in vivo*. In addition, Todorov *et al.* (27) reported that the bacteriocins of *L. sakei*, generally called sakacins, are more active against Gram-positive foodborne pathogens such as *Listeria monocytogenes*. Furthermore, LAB strains that show inhibitory activity against Gram-negative bacteria are more common in environments where co-evolution with other Gram-negative competitors has occurred (13).

The results of PCR detection of *L. sakei* L14 in faecal samples in this study suggest that its occurrence may only be transient in the chicken model. Previous studies confirm that most *Lactobacillus* strains can only temporarily colonise the chicken gut and some remain undetectable after infection (16, 23). As stated earlier, they are mostly diet-associated and may not be native to the gastrointestinal tract. The genome analysis of an *L. sakei* strain isolated from a French sausage revealed that it employs several strategies in order to grow on meat products. Its robustness enables it to outgrow competitors and survive the environmental

stresses associated with food processing such as high salt concentration, cold stress, and the presence of antimicrobial substances (13). These genomic characteristics make it well adapted to food-associated environments.

The absence of *Campylobacter* in the control group suggests that *C. jejuni* DC3 failed to colonise the gut, which could be due to the persistence of LAB and other bacteria there despite the antibiotic treatment prior to the challenge. The presence of *Campylobacter* in the co-infection group having been inconsistent suggests that it may not have been able to maintain its ability to colonise the gut, which could be attributed to the presence of *L. sakei* L14 and other LAB. While it was detected by PCR, this does not directly suggest that *L. sakei* did not have the ability to reduce the *Campylobacter* load, so further examination is recommended for a quantitative evaluation of the anti-*Campylobacter* activity exerted by *L. sakei in vivo*. The persistence of LAB across the groups despite antibiotic treatment suggests that an extended antibiotic treatment period may be necessary, in addition to variegation of the antibiotic cocktail administered, to ensure the elimination of all endogenous LAB.

In conclusion, the chicken intestine-isolated *L. sakei* L14 exhibited antagonistic activity against *C. jejuni* DC3 *in vitro*. The initial characterisation of the LAB isolate's anti-*Campylobacter* activity suggested the production of organic acids. Its *in vivo* activity, however, did not match the *in vitro* findings in that *C. jejuni* was still able to colonise the chicks, although inconsistently. This suggests that protective mechanisms other than organic acid production might be in play, given the complexity of the avian gut environment. The bacteriocinogenic potential of *L. sakei* L14 should be explored, considering that it has been isolated from the gut despite being widely associated with food products and therefore with muscle tissue. Its immunomodulatory ability also warrants further examination, as the isolate has the potential to be used as a chicken probiotic aimed at reducing *Campylobacter* carriage by poultry.

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