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RESEARCH ARTICLE

Salmonella enterica subsp. *enterica* serovar Typhimurium and *Lactobacillus* spp. interactions *in vitro* elicit improved antimicrobial production

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ABSTRACT

Antimicrobial resistance (AMR) is a global health crisis. Despite the drug discovery efforts, AMR is increasing, and discoveries are nearly nil. It is thus critical to design new strategies. Probiotics are tapped as alternatives to antibiotics for the treatment of gut-associated diseases. *Lactobacillus* species, common in food products, can inhibit the growth of gut pathogens. Here, we demonstrate the antimicrobial activities of *Lactobacillus* species – *Lactobacillus paracasei*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* are enhanced when cocultured with *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Cell-free culture supernatants (CFCS) from cocultures of *Lactobacillus* spp. and *Salmonella enterica* serovar Typhimurium more potently inhibit pathogen growth than their monoculture counterparts. Interestingly, we discovered that *Salmonella enterica* serovar Typhimurium could enhance the production of antimicrobials from *Lactobacillus* spp., most evident in *L. delbrueckii* subsp. *bulgaricus*. Also, *L. delbrueckii* subsp. *bulgaricus* CFCS upregulates key *Salmonella* virulence genes, *hilA* and *sipA*. Whether this increases *Salmonella*'s pathogenicity *in vivo* or reduces pathogen fitness and growth inhibition *in vitro* warrants further investigation. We propose that these probiotic isolates may be utilized for innovative natural food processing and preservation strategies to control *Salmonella* food contaminations. Importantly, our findings that *Salmonella* elicits an enhanced antimicrobial activity from *Lactobacillus* spp. provide evidence of a pathogen-mediated elicitation of antimicrobial production. Therefore, extending this phenomenon to other microbial interactions may help augment the strategies for drug discovery.

Keywords: Antimicrobial resistance; coculture; elicitation; probiotics; *Salmonella* infection.

INTRODUCTION

Nature provides science with a rich tapestry of natural products for drug discovery and development. Of the approved antibacterial agents from 1981 to 2019, 48% were obtained from natural products or derivatives (Newman & Cragg, 2020). Besides plants, microorganisms constitute a significant source of bioactive secondary metabolites with various biological functions (Pham *et al*., 2019). However, the traditional antibiotic discovery method by culturing pure bacterial cultures in the laboratory has two major obstacles that hinder the discovery process. First, standard laboratory microbial culture conditions do not guarantee bioactivity manifestation, and second, even if the compound is produced, the yield is insufficient to proceed with further undertaking.

The continuous exposure of microorganisms to various interactions or stimuli in their natural environment triggers bioactive secondary metabolites' expression for survival. Sequencing and genome mining show that microbes contain an array of biosynthetic gene clusters (BGCs) that allow bioactive compounds' production. However, most BGCs remain cryptic or silent when microbes are cultured in the laboratory due to the absence of environmental cues (Rutledge & Challis, 2015). Additionally, most compounds produced in the laboratory result in low yields, limiting the antibiotic discovery process.

These problems can be circumvented by mimicking the interactions *in vitro* to activate the silent BGCs and possibly increasing the extract yield through cocultivation or coculture methods (Yu *et al*., 2019; Nguyen *et al*., 2020). Cocultivation is primarily used to elucidate interactions of populations, such as antagonism, competition, and symbiosis (Goers *et al*., 2014). In the context of drug discovery, signaling molecules/soluble factors released by a microbe (challenger) may act as an elicitor for the target microbe (challenged isolate) to activate BGCs and secrete antimicrobial substances that can be harnessed for medicinal purposes (Goers *et al*., 2014; Zhuang *et al*., 2019).

Probiotics are live microorganisms that provide health benefits for humans and animals when consumed adequately (FAO/WHO, 2001; Hill *et al*., 2014). They have been shown to produce antimicrobial substances, such as bacteriocins, lactic acid, hydrogen peroxide, and organic acids, that allow them to competitively exclude pathogens in the gut, including some antibiotic-resistant species (Djadouni & Kihal, 2012; Chen *et al*., 2019). Because it is generally recognized as safe

(GRAS) for consumption, probiotics might be useful as an alternative for managing antibiotic resistance through cell-mediated treatment, particularly in gut-related infections where its activity is concentrated, such as *Salmonella* infection. In the Philippines, *Salmonella* is one of the leading causes of foodborne infections (Azanza *et al*., 2019), making *Salmonella* infection the leading cause of mortality among reported cases of intestinal infectious diseases (DOH, 2013). Antibiotics can control *Salmonella* infection, but their overuse and misuse accelerate the development of pathogen resistance, rendering frequently used antimicrobials ineffective in treating *Salmonella* infections (Holmberg *et al*., 1984; Velge *et al*., 2005; Ventola, 2015).

Despite the efforts to discover and develop more potent antibiotics, the rapid acceleration of antimicrobial resistance combined with the slow progression of the discovery prompts antibiotic exploration using alternative methods. Based on the idea that microbial interactions influence the synthesis of secondary metabolites, this study determined if the enteric pathogen *Salmonella* creates soluble components that can enhance or elicit the antimicrobial activity of probiotics.

MATERIALS AND METHODS

Bacteria and Culture Conditions

Previously isolated *Lactobacillus* spp. (*Lactobacillus delbrueckii* subspecies *bulgaricus* IRL 14-03*, Lactobacillus casei* IRL 14-02, and *Lactobacillus paracasei* IRL-14-01) from probiotic food products in the Philippines were cultured in DeMan-Rogosa-Sharpe (MRS; HiMedia, Mumbai, India) agar/broth medium at 37°C (Nicdao *et al*., 2020). The *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella enterica* serovar Typhimurium) SL1344 obtained from the Pathogen-Host-Environment Interactions Research Laboratory of the University of the Philippines, Diliman, Quezon City, Philippines and the clinical isolates of Enteropathogenic *E. coli* (EPEC), *Shigella* sp., and *Salmonella enterica* subsp. *enterica* serovar Typhi provided by Dr. Demetrio L. Valle Jr.† were cultured in Trypticase Soy Broth (TSB; HiMedia) at 37°C. The identities of the microbial isolates were determined by routine culture-based testing and confirmed with 16S *rRNA* gene sequencing. Working cultures of the bacteria listed above were prepared by washing the cells of fresh cultures with 1X Phosphate Buffered Saline Solution (PBS, pH 7.2), then adjusting the cell concentration to 1.5×10^8 CFU/mL through visual comparison with a 0.5 MacFarland's standard followed by absorbance reading (UV-Vis Spectrophotometer: EpochTM, Biotek, Vermont, USA) at 600 nm in the appropriate culture media.

Cell-to-Cell Interaction in Modified Coculture Media

A nutrient-rich coculture medium was prepared with modifications based on Abhisingha *et al*. (2018) protocol by mixing an equal proportion of MRS broth and TSB (MRS/TSB). Working inocula of *L. delbrueckii* subsp. *bulgaricus, L. casei, L. paracasei,* and *Salmonella enterica* serovar Typhimurium were prepared in MRS/TSB, as previously described. Three sets of tubes containing the modified medium were prepared for each probiotic isolate. The first set was inoculated with a probiotic species and *Salmonella enterica* serovar Typhimurium with a final cell concentration calculated to reach 1.0×10^5 CFU/mL. The second set contained probiotics alone (probiotic control), while the third was with *Salmonella enterica* serovar Typhimurium alone (test bacteria control). All tubes were incubated for 24 hours at 37°C. Serial dilution and aerobic plate count by pour plating were performed for all the setups. The first set of tubes was plated on *Salmonella-Shigella* Agar (SSA; HiMedia) and MRS agar. The second set was plated on MRS agar and the third on SSA. The SSA plates were incubated for 24 hours and MRS agar for 48 hours at 37°C. Statistical analyses of the counts were done to compare the cell concentration of probiotic isolate and test bacteria in cocultures and their monoculture counterparts.

Indirect Coculture

Working cultures of the *Lactobacillus* spp. isolates and *Salmonella* were prepared and resuspended in a modified nutrient-rich medium of MRS/Mueller Hinton Broth (MHB; HiMedia) separately. In a 12-well plate, one milliliter of 1.5×10^6 CFU/mL of each *Lactobacillus* isolate was dispensed separately. A cell culture insert (Falcon™; Germany) with a 0.40 µm pore size polyethylene terephthalate (PET) membrane bottom was submerged into the inoculated well. One milliliter of 1.5×10^8 CFU/mL of *Salmonella* was added to the cell inserts. Monoculture setups were prepared in other wells by adding a sterile medium into the cell insert submerged into the *Lactobacillus*-inoculated well. *Salmonella* control was also prepared by adding a sterile medium into the well and placing a cell culture insert containing *Salmonella.* The 12-well plate was incubated at 37°C for 18 hours without agitation. After incubation, culture media from the well and insert were aspirated, centrifuged at 12 000 × g for 10 minutes, and sterilized with a 0.2 µm Millipore™ syringe filter (Merck; Darmstadt, Germany) to collect the cell-free culture supernatants (CFCS). The coculture cell-free culture supernatants (cCFCS) and monoculture cell-free culture supernatants (mCFCS) were then tested against *Salmonella* using the microbroth culture method.

Direct Coculture

Fresh cultures of *Lactobacillus* isolates and *Salmonella enterica* serovar Typhimurium were prepared, washed, and resuspended in MRS/MHB coculture medium as described in the preparation of working cultures. The suspension of every *Lactobacillus* species was adjusted to 1.5×10^6 CFU/mL (Lb6, Lc6, Lp6). Three suspensions of *Salmonella enterica s*erovar Typhimurium containing 1.5×10^6 (St6), 1.5×10^7 (St7), and 1.5×10^8 (St8) CFU/mL were prepared separately. Each *Lactobacillus* isolate was cocultured with the three concentrations of *Salmonella* (designated as Lb6St6, Lb6St7, Lb6St8, etc.). Monocultures of *Lactobacillus* (Lb6, Lc6, Lp6) were also prepared in the coculture medium. All cultures were incubated at 37°C for 18 hours. The cCFCS and mCFCs were collected and tested against *Salmonella* using the microbroth culture method.

Induction with *Salmonella* Cell-Free Culture Supernatant

Salmonella cell suspension in MHB was prepared at a concentration of 1.5×10^8 CFU/ml. This suspension was dispensed to six separate tubes and incubated at 37°C for 18 hours without agitation. To obtain the cell-free culture supernatant, one tube was sacrificed every three hours, from the third hour until the eighteenth hour, and stored at -20°C until further use. The cell suspension of each *Lactobacillus* isolates were prepared in MRS broth, and the cell concentration was adjusted to 1.5×10^6 CFU/ml. Each of the six collected CFCS from *Salmonella* (St3h, St6h, St9h, St12h, St15h, St18h) was used to induce the antibacterial activity of 1.5×10^6 CFU/ml of each *Lactobacillus* isolate. The coculture tubes containing equal volumes of *Lactobacillus* spp. and *Salmonella* CFCS were incubated at 37°C for 18 hours without agitation. The induced cell-free culture supernatants (iCFCS) from every tube were collected and tested against *Salmonella* using the microbroth culture method.

Antimicrobial Activity Assessment

The collected CFCS from different coculture setups were tested against *Salmonella* using the microbroth culture method. In a 96-well plate, thirty microliters of CFCS were dispensed, followed by the addition of 270 uL of 1.5×10^6 CFU/mL of *Salmonella.* The control well was dispensed with 270 µl of 1.5×10^6 CFU/mL of *Salmonella* and 30 uL of sterile coculture medium. A blank

well was also prepared by dispensing 300 µL sterile coculture medium. The 96-well plate was incubated at 37°C with one minute of agitation every 30 minutes for six to seven hours or until the growth turbidity absorbance of the *Salmonella* control corresponds to ≈ 1.0 × 10^9 CFU/mL. The absorbance was measured using UV-Vis spectrophotometry at 600nm.

Gene Expression Analysis

Working cultures of the *Lactobacillus* isolates, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Typhi, *Shigella* sp., Enteropathogenic *E. coli* (EPEC), and *E. coli* ATCC 25922 were prepared in MRS/MHB media as previously described. In a 12-well plate, one milliliter of 1.5 × 10^6 CFU/mL of each working culture was inoculated in separate wells. Another well was inoculated with one milliliter of sterile MRS/MHB medium. A cell culture insert was placed in all wells, followed by adding one milliliter of 1.5 × 10^8 CFU/mL of *Salmonella enterica s*erovar Typhimurium. The plate was incubated at 37°C for 18 hours without agitation, and *Salmonella enterica* serovar Typhimurium cells were collected and processed for RNA extraction using ZR Bacterial RNA Microprep (Zymo Research Corp., California, USA). The collected RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, California, USA). The RNA, at 10ng/µL concentration, was used to analyze the gene expression of *hil*A and *sip*A virulence genes of *Salmonella*. The relative quantification of *hil*A and *sip*A expression of *Salmonella* was analyzed using the primer pairs *hil*A/F (5'-CGGAACGTTATTTGCGCCATGCTGAGGTAG-3') and *hil*A/R (5'-GCATGGATCCCCGCCGG CGAGATTGTG-3') (Pathmanathan and Sa 2003) and *sip*A/F (5'-CGGCTTCACATTCACAA-3') and *sip*A/R (5'-CGGGCTCTTTCGT TCA-3') (Hassuny *et al*., 2015). Quantitative PCR (qPCR) mix was prepared using SsoFast EvaGreen Supermix (Bio-Rad Laboratories) following the manufacturer's recommendations. Cycling conditions for qPCR include enzyme activation at 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 5 seconds, annealing/extension at 65°C for 5 seconds, and a final melt curve at 65-95°C in 0.5°C increment every 2 seconds (Bio-Rad Laboratories). The expression analysis was standardized using *16S rRNA* as the reference gene as analyzed using primers 16S 514F (5'-GCAATTGACGTTACCCGCA GAA-3') and 16S 642R (5'- GGATTTCAC ATCCGACTTGACA-3') for both treated and untreated *Salmonella* culture.

Statistical Analysis

The data from replicated experiment trials were analyzed using IBM SPSS version 23.0 for Mac. Mann-Whitney U test and Tukey's-b were used to determine the statistical significance between samples at $p \leq 0.05$.

RESULTS

Probiotic isolates reduce recoverable *Salmonella enterica serovar Typhimurium* cells in coculture growth while maintaining unperturbed growth

The ability of *Lactobacillus* spp. isolates to antagonize the growth of a gut pathogen, *Salmonella enterica* serovar Typhimurium, was investigated by coculturing each probiotic isolate, *Lactobacillus* species: *L. delbrueckii* subsp. *bulgaricus*, *L. casei,* and *L paracasei,* with *Salmonella enterica* serovar Typhimurium in a modified coculture media.

Consistent with previous reports that some lactic acid bacteria (LAB) can inhibit *Salmonella* growth (Kim *et al*., 2004; Makras & De Vuyst, 2006; Ishikawa *et al*., 2010), a decrease in the recoverable viable colonies of *Salmonella enterica* serovar Typhimurium from probiotics-pathogen cocultures was observed (Figure 1). When cocultured with probiotic isolates, the recoverable CFUs of *Salmonella enterica* serovar Typhimurium were lower than the CFUs from the control *Salmonella enterica* serovar Typhimurium monoculture grown in the same culture medium. From a recoverable cell count of 2.29 × 10^9 CFU/mL in the pathogen monoculture control, a one-log to a two-log-fold reduction in recoverable CFUs in all coculture setups was observed. Although the CFU trend of CFUs decreased in all coculture setups, the results were statistically not significant at $p \leq 0.05$. Notably, *Lactobacillus delbrueckii* subsp. *bulgaricus* had the highest growth inhibitory effect on *Salmonella enterica* serovar Typhimurium, reducing recoverable CFU/mL to 3.67 × 10^7 CFU/mL.

Interestingly, the number of viable cells of *Lactobacillus* spp. isolates were not significantly affected when cocultured with *Salmonella enterica* serovar Typhimurium. The results thus showed that all the *Lactobacillus* spp. isolates can inhibit the growth of *Salmonella enterica* serovar Typhimurium when cocultured *in vitro* while maintaining their growth relatively unperturbed by the pathogen.

Indirect cocultures of probiotic isolates and *Salmonella enterica* serovar Typhimurium produce cell-free culture supernatants (cCFCS) with more potent inhibitory activity than their probiotic monoculture (mCFCS) counterparts

Following the cell-to-cell interaction study between *Lactobacillus* isolates and *Salmonella enterica* serovar Typhimurium, parallel experiments of the *Salmonella enterica* serovar Typhimurium

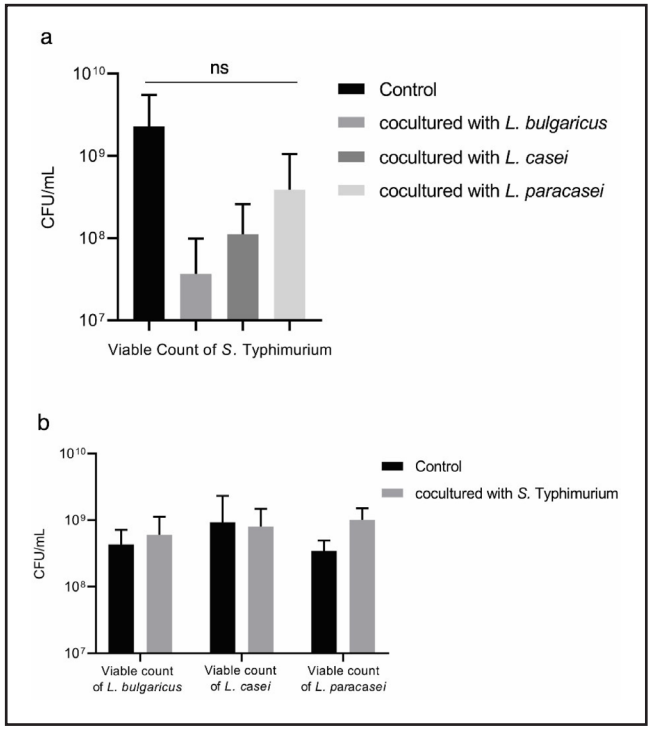

Figure 1. Recoverable viable colony forming units (CFU/mL) of *Salmonella enterica* serovar Typhimurium and *Lactobacillus* spp. from cocultures. Probiotic *Lactobacillus* spp. isolates (*L. delbrueckii* subsp. *bulgaricus*, *L. casei,* and *L. paracasei*) were cocultured with the *Salmonella enterica* serovar Typhimurium enteric pathogen in a modified nutrient-rich coculture medium for 24 hours at 37°C. The recoverable CFUs were quantified by plating the cocultures on modified selective media. (a) The growth of enteric pathogen *Salmonella enterica* serovar Typhimurium recovered from cocultures. (b) The growth of *Lactobacillus* spp. probiotic isolates recovered from coculture. The values presented are average counts from three independent experiments; error bars represent standard deviations. Values are not significantly different at $p \leq 0.05$.

growth inhibitory effects of cell-free culture supernatants from monocultures of *Lactobacillus* spp. (mCFCS) and *Lactobacillus* spp. and *Salmonella enterica* serovar Typhimurium cocultures (cCFCS) were performed by restricting direct cell interaction using a cell culture insert. The growth of untreated *Salmonella enterica* serovar Typhimurium at A_{600} of 0.616 was significantly reduced to 0.559 ($p \leq 0.001$) by treatment with mCFCS of *L. delbrueckii* subsp. *bulgaricus* (Lb6), to 0.448 ($p \leq 0.001$) by mCFCS of *L. casei* (Lc6), and to 0.436 ($p \leq 0.001$) by *L. paracasei* (Lp6). Interestingly, these reductions in *Salmonella enterica* serovar Typhimurium growth were further enhanced when cCFCS were used (Figure 2). Growth turbidity was further reduced to A_{600} of 0.495 ($p \leq 0.001$) with coculture of *Salmonella enterica s*erovar Typhimurium with *L. delbrueckii* subsp. *bulgaricus* (Lb6/St8), to 0.388 ($p \leq 0.01$) with *L. casei* (Lc6/St8), and to 0.398 ($p \leq 0.01$) with *L. paracasei* (Lp6/St8).

The growth inhibitory effects of cCFCS generated from the indirect coculture were significantly higher than mCFCS collected from three probiotic isolates, suggesting an exchange of soluble factors between *Salmonella enterica* serovar Typhimurium and the *Lactobacillus* spp., which enhances the antagonistic activity of the probiotic isolates against this pathogen.

Increasing cell concentration of *Salmonella enterica* serovar Typhimurium used in coculture with the probiotic isolates increases the growth-inhibitory effect of cCFCS produced

To ascertain that *Salmonella enterica* serovar Typhimurium elicits an increase in antagonistic activity of *Lactobacillus* spp. in a concentration-dependent manner, the direct coculture method was conducted with increasing doses of *Salmonella enterica* serovar Typhimurium. *L. delbrueckii* subsp. *bulgaricus* mCFCS demonstrated a 0.498 to 0.147 ($p \leq 0.05$) significant decrease of *Salmonella enterica* serovar Typhimurium A_{600} growth turbidity (Figure 3a) compared to untreated *Salmonella enterica* serovar Typhimurium control. This activity was enhanced when cCFCS was used – furthermore, the anti-*Salmonella enterica* serovar Typhimurium activity increased in a *Salmonella enterica* serovar Typhimurium dose-dependent manner. The cCFCS from *L. delbrueckii* subsp. *bulgaricus* cocultured with *Salmonella enterica* serovar Typhimurium concentration of 1.5×10^6 CFU/mL (Lb6St6) resulted in further decrease in A_{600} to 0.166 ($p \leq 0.05$), while the pathogen concentration of 1.5×10^7 CFU/mL (Lb6St7) was lowered to 0.103 ($p \leq 0.05$), and at 1.5×10^8 CFU/mL (LbSt8) is at its lowest of 0.095 ($p \leq 0.05$) (Figure 3a).

L. casei mCFCS also significantly reduced the *Salmonella enterica* serovar Typhimurium growth from 0.511 to 0.316 ($p \leq 0.05$) (Figure 3b). Increasing concentrations of *Salmonella enterica* serovar Typhimurium in cocultures improved the growth inhibitory effects of cCFCS with a dose-dependent decrease of A_{600} to 0.282 ($p \leq 0.05$), 0.225 ($p \leq 0.05$), up to 0.203 ($p \leq 0.05$) at *Salmonella enterica* serovar Typhimurium doses of 1.5×10^6 CFU/

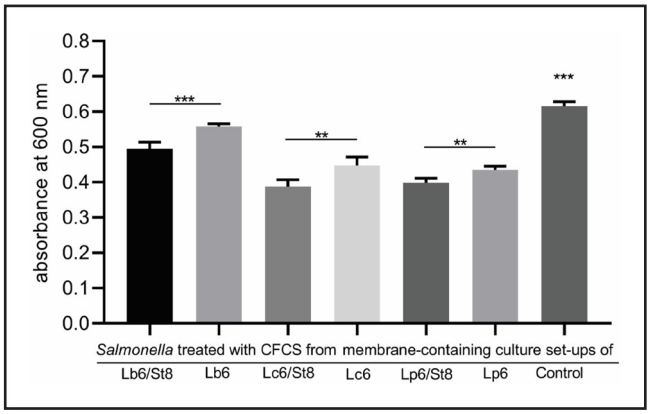

Figure 2. Comparison of the growth of *Salmonella enterica s*erovar Typhimurium treated with cell-free culture supernatants from indirect cocultures (cCFCS) and monocultures (mCFCS). CFCS were collected from cocultures of 1×10^6 CFU/mL *L. delbrueckii* subsp. *bulgaricus* and 1×10^8 CFU/mL *Salmonella enterica* serovar Typhimurium (Lb6/St8), 1×10^6 CFU/mL *L. casei* and 1×10^8 CFU/mL *Salmonella enterica s*erovar Typhimurium (Lc6/St8), and 1×10^6 CFU/mL *L. paracasei* and 1×10^8 CFU/mL *Salmonella enterica* serovar Typhimurium (Lp6/St8) that were incubated for 18 hours at 37°C. CFCSs were added to *Salmonella enterica* serovar Typhimurium inoculum and incubated for six hours at 37°C. In a parallel experiment, the growth of *Salmonella enterica* serovar Typhimurium was treated with culture supernatant from 1×10^6 CFU/mL starting inoculum of probiotic monoculture (mCFCS) (LB6, LC6, and Lp6). The growth of *Salmonella enterica* serovar Typhimurium on broth cultures was assessed by reading absorbance values at 600 nm. The values presented are average readings from three experiments. Values significantly different from the control are indicated by asterisk(s) (* at $p \leq 0.05$; ** at $p \leq 0.01$; *** at $p \leq 0.001$).

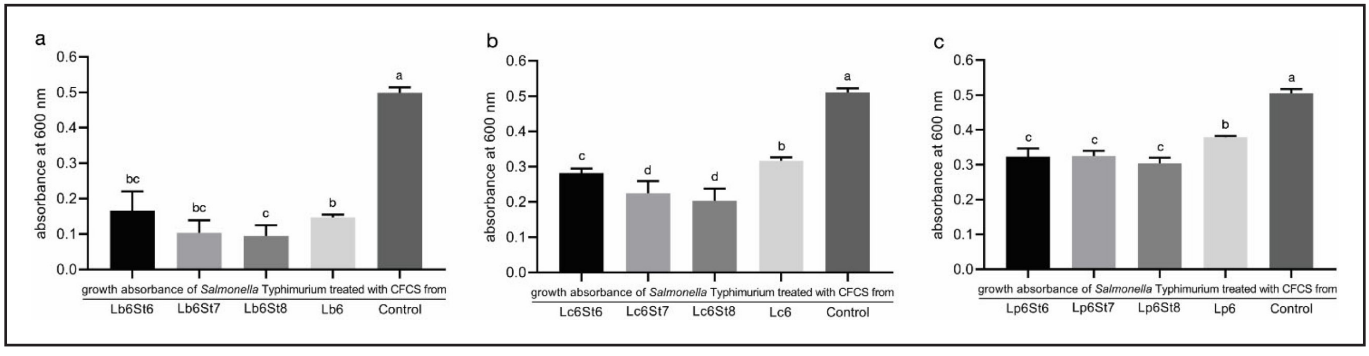

Figure 3. Growth absorbance of *Salmonella* treated with cell-free culture supernatants collected from direct cocultures (cCFCS) of *Lactobacillus* spp. and different cell concentrations of *Salmonella enterica* serovar Typhimurium. CFCS were collected from cocultures of either *L. delbrueckii* subsp. *bulgaricus* (Lb6), *L. casei* (Lc6), or *L. paracasei* (Lp6) and increasing doses of *Salmonella enterica* serovar Typhimurium (St6, /St7, or /St8). Collected cCFCS were tested against *Salmonella enterica* serovar Typhimurium, and corresponding probiotic monoculture (mCFCS) (Lb6, Lc6, and Lp6) were compared. The growth of *Salmonella enterica* serovar Typhimurium in broth cultures was assessed by reading absorbance values at 600 nm. (a) The growth of *Salmonella enterica* serovar Typhimurium treated with cCFCS from cocultures with *L. delbrueckii* subsp. *bulgaricus,* (b) the growth of *Salmonella enterica* serovar Typhimurium treated with cCFCS from cocultures with *L. casei*, and (c) the growth of *Salmonella enterica* serovar Typhimurium treated with cCFCS from cocultures with *L. paracasei*. The values presented are average readings from three experiments; error bars represent standard deviations. Values that are significantly different from the control are indicated by a different letter ($p \leq 0.05$).

mL (Lc6St6), 1.5 × 10^7 CFU/mL (Lc6St7), and 1.5 × 10^8 CFU/mL, respectively (Figure 3b).

A similar trend was observed in *L. paracasei* with a decrease in growth turbidity absorbance reading of 0.505 to 0.379 ($p \leq 0.05$) (Figure 3c) when *Salmonella enterica* serovar Typhimurium was treated with mCFCSalmonella This inhibition was further enhanced when coculture supernatants from *L. paracasei* cocultured with *Salmonella enterica* serovar Typhimurium (cCFCS) were used. The absorbance readings of the growth turbidity of *Salmonella enterica* serovar Typhimurium decreased further to a range of 0.304 to 0.343 ($p \leq 0.05$). However, unlike with *L. delbrueckii* subsp. *bulgaricus* and *L. casei,* the inhibitory activity of *L. paracasei* in cocultures did not significantly demonstrate a *Salmonella enterica* serovar Typhimurium dose-dependency at the aforementioned microbial range (Figure 3c).

mCFCS from *Salmonella enterica* serovar Typhimurium elicits the production of inhibitory growth factors in *Lactobacillus* spp. cultures

The interesting findings that CFCS from cocultures of *Salmonella enterica* serovar Typhimurium and *Lactobacillus* spp. (cCFCS) more potently inhibits the growth of *Salmonella enterica s*erovar Typhimurium led to our hypothesis that *Salmonella enterica* serovar Typhimurium releases soluble factors that elicit the production of antagonistic activity from *Lactobacillus* spp. To test this hypothesis, mCFCS of *Salmonella enterica* serovar Typhimurium were collected at different time points post-inoculation and used to induce monocultures of *Lactobacillus* spp. to produce anti-*Salmonella enterica* serovar Typhimurium activity. Consistent with the preceding data sets, mCFCS of *L. delbrueckii* subsp. *bulgaricus* (Lb) significantly decreased the growth absorbance (A_{600}) of *Salmonella enterica* serovar Typhimurium untreated control from 0.514 to 0.199 ($p \leq 0.05$). It was also found that inducing a monoculture of *L. delbrueckii* subsp. *bulgaricus* with *Salmonella enterica* serovar Typhimurium CFCS collected at different time points of its growth enhanced the anti-*Salmonella* activity of *L. delbrueckii* subsp. *bulgaricus* (Figure 4a). The iCFCS from *L. delbrueckii* subsp. *bulgaricu*s induced using the supernatant of *Salmonella enterica* serovar Typhimurium collected at three hours post-inoculation (LbSt3h) significantly reduced the A_{600} to 0.111 ($p \leq 0.05$) compared to uninduced mCFCS. Treatments of *L. delbrueckii* subsp. *bulgaricus* cultures induced with 6 hours, 9 hours, and 12 hours of *Salmonella enterica s*erovar Typhimurium culture supernatant (LbSt6h, LbSt9h, LbSt12h) also significantly decreased the A_{600} ranging from 0.111 to 0.119 ($p \leq 0.05$). Treatment with iCFCS induced with 15-hour *Salmonella enterica* serovar Typhimurium supernatant (LbSt15) was 0.143 ($p \leq 0.05$) and at 18-hours (LbSt18) 0.169 ($p \leq 0.05$) (Figure 4a).

The growth of untreated *Salmonella enterica* serovar Typhimurium control also decreased from A_{600} of 0.514 to 0.372 ($p \leq 0.05$) when treated with monoculture supernatant of *L. casei* (Lc). A further decrease was observed when *Salmonella enterica* serovar Typhimurium was treated with iCFCS from *L. casei* induced with *Salmonella enterica* serovar Typhimurium supernatant collected at different hours post-inoculation referred to as LcSt3h, LcSt6h, LcSt9h, LcSt12h, LcSt15h, LcSt18h. The growth turbidity A_{600} values ranged from 0.327 to 0.354 ($p \leq 0.05$), which were not statistically different between treatments but significantly lower than the values of untreated control and mCFCS-treated *Salmonella enterica* serovar Typhimurium cultures (Figure 4b).

L. paracasei monoculture CFCS inhibited *Salmonella enterica* serovar Typhimurium growth as reflected by the decrease in A_{600} growth turbidity values of untreated control from 0.643 to 0.502 ($p \leq 0.05$) upon treatment with *L. paracasei* monoculture CFCS (Lp). *Salmonella enterica* serovar Typhimurium growth inhibition was also enhanced in cultures treated with iCFCS from *L. paracasei* (LpSt3h, LpSt6h, LpSt9h, LpSt12h, LpSt15h, LpSt18h) with A_{600} ranging from 0.374 to 0.391 ($p \leq 0.05$), which were not statistically different among different time points but were all significantly lower than the control and monoculture setup (Figure 4c).

Altogether, the data demonstrated that *Salmonella enterica* serovar Typhimurium can elicit the production of more potent antagonistic soluble factors from *Lactobacillus* spp. and that this phenomenon is most evident in *L. delbrueckii* subsp. *bulgaricus*. These findings suggest that this probiotic isolate can be induced to respond more vigorously to antagonize the growth of an enteric pathogen.

***Salmonella enterica* serovar Typhimurium-*L. delbrueckii* subsp. *bulgaricus* coculture interaction increases *hilA* and *sipA* gene expression in *Salmonella enterica* serovar Typhimurium**

To investigate the effects of probiotics and pathogen interactions on key regulatory and pathogenicity gene expressions of *Salmonella enterica* serovar Typhimurium, the transcriptional regulator gene *hilA* of *Salmonella* Pathogenicity Island-1 (SPI-1)

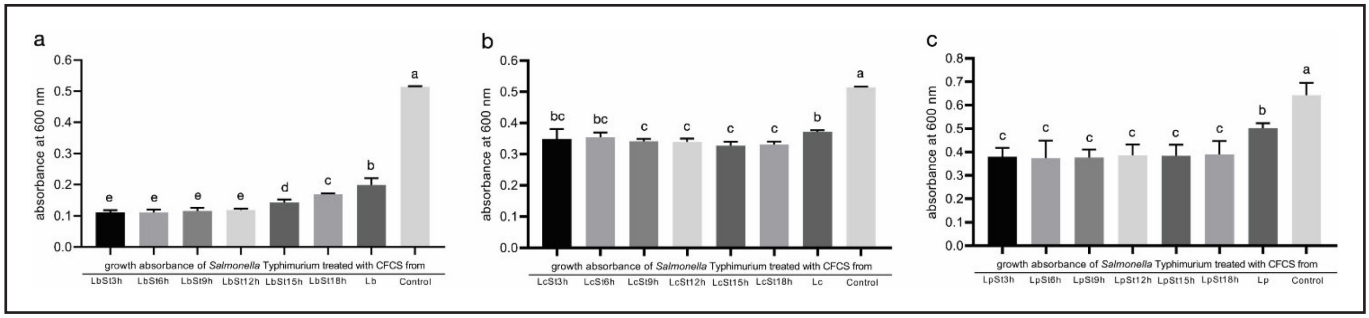

Figure 4. Growth of *Salmonella enterica s*erovar Typhimurium treated with induced cell-free culture supernatants (iCFCS) collected from *Lactobacillus* spp. monocultures that were induced with CFCS from monocultures of *Salmonella enterica s*erovar Typhimurium collected at different time points. CFCS from *Salmonella enterica* serovar Typhimurium monocultures incubated for 3 hours (St3h), 6 hours (St6h), 9 hours (St9h), 12 hours (St12h), 15 hours (St15h), and 18 hours (St18h) at 37°C were collected and used to treat 1 × 10^6 CFU/mL inoculum of *Lactobacillus* spp. monocultures to induce the production of inhibitory growth factors. Cell-free culture supernatants from induced probiotic cultures (iCFCS) were used to treat *Salmonella enterica* serovar Typhimurium and incubated for six hours at 37°C. The growth of *Salmonella enterica* serovar Typhimurium on broth cultures was assessed by reading absorbance values at 600 nm. (a) Growth of *Salmonella enterica* serovar Typhimurium in iCFCS from *L. delbrueckii* subsp. *bulgaricus*, (b) Growth of *Salmonella enterica* serovar Typhimurium in iCFCS from *L. casei,* and (c) Growth of *Salmonella enterica* serovar Typhimurium in *L. paracasei.* The values presented are average readings from three experiments. Values that are significantly different from the control are indicated by a different letter ($p \leq 0.05$).

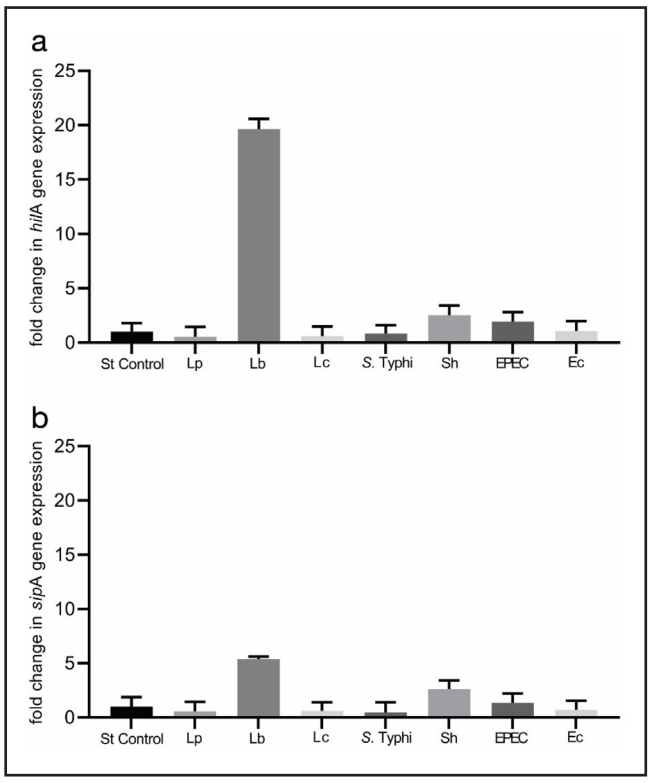

Figure 5. Induction of *hilA* and *sipA* gene expression in *Salmonella enterica* serovar Typhimurium. Gene expression of *hilA and sipA* quantified from *Salmonella enterica* serovar Typhimurium cocultured for 18 hours with different probiotic *Lactobacillus* isolates and some gut pathogens physically separated by a semi-permeable membrane. Expression of the *hil*A (a) and *sip*A (b) genes of *Salmonella enterica* serovar Typhimurium when cocultured with *L. paracasei* (Lp)*, L. delbrueckii* subsp. *bulgaricus* (Lb)*, L. casei* (Lc)*, Salmonella enterica* serovar Typhi (STyphi), *Shigella* sp. (Sh), Enteropathogenic *E. coli* (EPEC) or *E. coli* ATCC 25922 (Ec). The fold change in gene expression is compared with untreated *Salmonella enterica* serovar Typhimurium (St Control) with a reference value of 1.0. The values presented are averaged from 3 experiments.

and SPI-1 effector gene *sipA* expressions were evaluated by qRT-PCR in coculture experiments. Interestingly, these two genes were upregulated when *Salmonella enterica* serovar Typhimurium was cocultured with *L. delbrueckii* subsp. *bulgaricus*. An approximately 20-fold increase in the *hilA* gene expression (Figure 5a) and a 5-fold increase in the *sipA* gene expression (Figure 5b) were observed. These genes were not significantly affected by *L. casei* and *L. paracasei*. The coculture of *Salmonella enterica* serovar Typhimurium with other enteric bacteria – *Salmonella* Typhi, *Shigella* sp., EPEC, and *E. coli* ATCC 25922 – also did not induce pronounced gene modulation except for *Shigella*, for which a modest increase in gene expression was observed.

These findings demonstrated the interaction of *Salmonella enterica* serovar Typhimurium with *L. delbrueckii* subsp. *bulgaricus* elicit active responses from the pathogen that may give insights into how this probiotic can inhibit pathogen growth.

DISCUSSION

In this study, we investigated three *Lactobacillus* species isolated from probiotic food products in the Philippines for their ability to control gut pathogens, particularly *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Interestingly, while testing for the anti-*Salmonella* activity of the *Lactobacillus* isolates, we uncovered an important interaction between *Salmonella* and *Lactobacillus delbrueckii* subsp. *bulgaricus.* Our data suggest that *Salmonella* secretes a soluble factor that enhances the potency of *L. delbrueckii* subsp. *bulgaricus* to produce antagonistic factors against *Salmonella.* Furthermore, in contrast to other *Lactobacillus* species that we tested – *L. casei* and *L. paracasei,* we found that *L. delbrueckii* subsp. *bulgaricus* was able to upregulate *hil*A and *sip*A genes in *Salmonella*. Several studies have observed the anti-*Salmonella* activity of *Lactobacillus* species. However, their activities are strain-specific (Golowczyc *et al*., 2007; Yang *et al*., 2014; Gómez *et al.*, 2016). Furthermore, to our knowledge, this may be the first report describing that *Salmonella* can elicit a more potent antibacterial activity from *Lactobacillus* species in the cell-free culture supernatant (CFCS).

Our study's novel finding is *Salmonella*'s ability to elicit an antagonistic activity from *L. delbrueckii* subsp. *bulgaricus* potentiating its anti-*Salmonella* activity (Figures 2 and 3). Although *L. delbrueckii* subsp. *bulgaricus* monoculture exhibited a natural or innate inhibitory activity against *Salmonella enterica s*erovar Typhimurium, its coculture with *Salmonella,* either in the presence or absence of cell-to-cell contact, demonstrated a more potent antagonistic activity (Figures 1 and 2). The amelioration may have resulted from a direct cell-to-cell interaction (Figure 3) between the two species or the exchange of their metabolites (Figure 2). During the interaction, membrane-bound molecules or secreted compounds may trigger a cell signaling network that directs the expression of genes necessary for growth and survival. Two proposed mechanisms have been inferred: (1) the isolates, especially *L. delbrueckii* subsp. *bulgaricus,* sense cues from *Salmonella enterica* serovar Typhimurium, amplifying the production and secretion of the innately produced antimicrobial compounds, and (2) the signaling cues released by *Salmonella enterica* serovar Typhimurium promoted the expression of other biosynthetic gene clusters (BGCs) in *L. delbrueckii* subsp. *bulgaricus*, encoding a more potent antimicrobial compound that can either be bacteriostatic or bactericidal. Microbial interactions may induce the test bacteria to diversify their secreted metabolome or potentiate their secondary metabolite production. Our finding that *Salmonella* potentiates the isolates' antibacterial activity was similarly observed in our biological models of cell-cell interaction using diffusible molecules or direct cell contact. This phenomenon was demonstrated in microbial communities from similar soil habitats with 146 phylogenetically different bacteria. Their pairwise combination demonstrated 46% antimicrobial activity compared to 33% in monocultures. There was also interaction-mediated suppression of inhibitory activity in 22% of all combinations (Tyc *et al*., 2014). In species of *Streptomyces,* when *Salmonella coelicolor* interacted with other actinomycetes, each interaction triggered a unique secondary metabolite profile (Traxler *et al*., 2013). The displayed competitive interactions produced antimicrobial compounds only in the presence of competing species, the consequence of which is the inhibition of other cells. Our model of cell-to-cell interaction may also be observed in *Pseudomonas aeruginosa.* The production of its antimicrobial compound is enhanced in response to *N*-Acetylglucosamine peptidoglycan. This suggests that *P. aeruginosa* monitors other bacteria in its environment by recognizing exogenous peptidoglycan (Korgaonkar & Whiteley, 2011). There are also rare accounts of this competition-induced potentiated antimicrobial activity in probiotics. Tong *et al*. (2012) reported competition between the probiotic *Lactococcus lactis* and the dental plaque-inducing *Streptococcus mutans.* Diffusible molecules from the metabolites of *Salmonella mutans* enhanced the bacteriocin nisin expression in *L. lactis,* which resulted in the inhibition of the former (Tong *et al*., 2012). These studies may explain how *Salmonella* potentiated the antagonistic activity of the isolates, particularly *L. delbrueckii* subsp. *bulgaricus.*

Probiotic involvement in the modulation of gene expression in *Salmonella* has been previously studied (Das *et al*., 2013; Yang *et al*., 2014; Tanner *et al*., 2016), but little is known about the probiotics' transcriptome as they interact or when cocultured with enteric pathogens. Given that microbial interaction in nature is always complex, we inferred that *Salmonella-Lactobacillus* interaction does not only affect *Salmonella*. The consequences of their interaction are reciprocal, and the modulation of gene expression is bilateral. In our study, a direct relationship was observed between the increasing cell concentrations of *Salmonella* and the inhibitory activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Figure 3). Additionally, the potentiated antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* can be triggered in the absence of live cells of *Salmonella*. This suggests that the metabolites secreted during logarithmic and stationary phases of *Salmonella* growth can elicit antimicrobial activity. When bacteria interact, they may sense general cues of potential danger or specific threats from competitors; they employ distance-dependent danger sensing of volatile organic compounds, diffusible molecules, and direct contact (Westhoff *et al*., 2017).

As the three *Lactobacillus* isolates may use soluble factors to inhibit *Salmonella enterica* serovar Typhimurium, the *L. delbrueckii* subsp. *bulgaricus* isolate may use an additional species-specific mechanism that capacitates it to inhibit the growth of *Salmonella enterica* serovar Typhimurium at a greater magnitude than the other two isolates. In contrast to other *Lactobacillus* species tested in this study – *L. casei* and *L. paracasei, L. delbrueckii* subsp. *bulgaricus* upregulated *hil*A and *sip*A genes in *Salmonella* by 20-fold and 5-fold, respectively. We propose that the upregulation of key virulence genes might be another mechanism by which this isolate controls *Salmonella* growth (Figure 5).

The *Salmonella* pathogenicity island 1 (SPI-1) encodes several effector proteins necessary to invade the epithelial cells (Fàbrega & Vila, 2013). The transcription factor encoded in SPI-1, the *hil*A gene, is responsible for controlling downstream genes in the SPI-1, which are necessary for the disruption and invasion of the gut barrier system (Bajaj *et al*., 1996; Fàbrega & Vila, 2013). It regulates the *prg/org* and *inv/spa* operons that encode the formation of invasion-associated type III secretion system (T3SS-1) (Ellermeier & Slauch, 2007; Fʁbrega & Vila, 2013) and the *sic/sip* operons that encode effector proteins, which impair the tight junctions responsible for intestinal barrier integrity (Boyle *et al*., 2006; Fàbrega & Vila, 2013). Importantly, *hilA* controls the gene *sip*A (Galan, 1996; Fʁbrega & Vila, 2013), which encodes for a protein that mediates membrane ruffling of the host cell through actin binding, bundling, and polymerization leading to the phagocytosis of the *Salmonella* cells (Agbor & McCormick, 2011). Along with other genes in the *Salmonella* Pathogenicity Island-1, *hilA* and *sipA* have significant roles in *Salmonella* infection.

Reports have described how *Lactobacillus* spp. modulate SPI-1 genes to control *Salmonella'*s invasion of host epithelial cells by downregulating *hilA* and the downstream genes (De Keersmaecker *et al*., 2005; Bayoumi & Griffiths, 2010; Yang *et al*., 2014), but some reported otherwise. Tanner *et al*. (2016) explored the global transcription response of *Salmonella enterica* serovar Typhimurium N-15 as it interacts with *Bifidobacterium thermophilum* RBL6 in coculture. They found that many virulence genes in *Salmonella* N-15 were highly expressed, including the complete T3SS-1 genes and the T3SS-1 regulator, *hilA* (Tanner *et al*., 2016). However, this would contradict many studies showing reduced invasion capacity of *Salmonella* as evident in the downregulation of the virulence genes (De Keersmaecker *et al*., 2006; Bayoumi & Griffiths, 2010; Yang *et al*., 2014).

So how can an increased virulence gene expression lead to reduced cell proliferation and infection rate? While the virulence genes of *Salmonella* N-15 were highly expressed, its cell count was reduced upon coculture with *Bifidobacterium* RBL67 (Tanner *et al*., 2016). Similarly, when the *L. delbrueckii* subsp. *bulgaricus* isolate was cocultured with *Salmonella enterica* serovar Typhimurium indirectly, the *hilA* and *sipA* virulence genes of *Salmonella* were overexpressed. In a host-pathogen scenario, a subpopulation of *Salmonella* expresses T3SS-1 to trigger cell host invasion and pro-inflammatory response (Sturm *et al*., 2011; Diard *et al*., 2013). However, T3SS-1-expressing *Salmonella* (T^+) cells are not involved in the invasion of host cells; they only lay the groundwork for an actual infection. T^+ cells typically form 20-200 T3S apparatuses and approximately $3–10 \times 10^4$ effector proteins (Sturm *et al*., 2011), which is costly to cells; their growth becomes retarded, they become less fit, and they are easily killed (Ackermann *et al*., 2008; Sturm *et al*., 2011; Diard *et al*., 2013). Thus, they exhibit self-destructive cooperation to benefit other subpopulations of *Salmonella* (Ackermann *et al*., 2008; Diard *et al*., 2013).

Although this study did not explore the global transcriptome of *Salmonella enterica* serovar Typhimurium, we propose two possible scenarios underlying our results. First, the induction of *hil*A and *sip*A expression in *Salmonella* may lead to the formation of T^+ cells. We hypothesize that as cells of *Salmonella* and *L. delbrueckii* subsp. *bulgaricus* interact in the experimental setup, the *L. delbrueckii* subsp. *bulgaricus* isolate induces the formation of T^+ cells, which are less fit and easily killed by the soluble factors that *L. delbrueckii* subsp. *bulgaricus* produces. While the results of the coculture experiments might partially substantiate this premise, we do not discount other possibilities, including the survival of other pathogen subpopulations. Second, we note that the upregulation of *hilA* may significantly increase the gut epithelial lining invasion by the pathogen. This can be exploited as an evasion mechanism by *Salmonella enterica* serovar Typhimurium against the antimicrobial assault of *L. delbrueckii* subsp. *bulgaricus*. Therefore, further studies are needed to test these hypotheses to gain more insights into the impact of these microbial interactions *in vivo*.

CONCLUSION

The rise of antimicrobial resistance renders available antibiotics ineffective against infectious bacteria. At present, the diarrheal diseases caused by enteric pathogens need to be addressed by exploring alternative forms of management to address pathogens' antimicrobial resistance. Probiotic microorganisms produce antimicrobial substances that can be explored for therapeutic purposes. One mechanism by which probiotic microorganisms perform this is through the competitive exclusion of pathogens. In nature, microorganisms communicate and react by releasing signaling cues that can be antagonistic or synergistic, among others. In this study, we found that *Salmonella* could elicit the production of more potent antimicrobial factors from probiotic isolates in response to the presence of live cells of *Salmonella* or through its metabolites. These soluble factors can be capitalized as a *Salmonella* inhibitor, especially during a non-Typhoidal diarrheal disease.

Considering the probiotics' status as generally regarded as safe (GRAS), our findings have at least two potential applications. First is the enhancement of antimicrobial compound production by *Lactobacillus* spp. against *Salmonella* infection *in vitro*. The second application uses *Lactobacillus* spp. for innovative natural food processing and preservation strategies to control *Salmonella* contamination. It may reduce the risk of infection caused by ingesting contaminated food products.

Although the *in vitro* results for the potentiation of inhibitory activity of *L. delbrueckii* subsp. *bulgaricus* by *Salmonella* is promising, the translation of the experiment to an *in vivo* setting

warrants further investigations. Microbial interactions simulated in a laboratory setup differ significantly in an *in vivo* setting with more variables. Further research is needed to validate the efficacy of probiotic strains for infectious disease treatment. Therefore, we may prevent their adverse risk and maximize health and economic benefits by carefully investigating probiotic microorganisms' biological activities.

Importantly, our study provided valuable insights into how microbial interactions could be mined for elicitors of antimicrobial substances that may be tapped for improving their production. As we struggle to increase our arsenal of strategies for controlling infectious diseases, we believe our study paved a potential new avenue for enhancing antimicrobial production.

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This work is dedicated to the late father of Michael Angelo Nicdao, Mr. Orencio "Orange" Vicente Nicdao.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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