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A cocktail of *Lactobacillus* species controls *Salmonella* infection and maintains animal productivity in poultry farming

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Poultry chickens are common carriers of the gut pathogen, *Salmonella.* They can transmit this pathogen to humans through contaminated eggs and meat. *Salmonella* can be controlled in poultry farms using antibiotics. But because of the unregulated use of antibiotics in poultry farming that contributes to the emergence of antibiotic resistance, alternatives to antibiotics are being sought. Therefore, this study determined whether food product isolates of *Lactobacillus* spp., which were previously found to inhibit *Salmonella in vitro,* could be exploited to control *Salmonella* infection in Cobb broiler chickens. Initially, the *Lactobacillus* spp. were tested on small animals—BALB/c mice. *L. paracasei* IRL14-01, *L. casei* IRL14-02, and *L. delbrueckii* subsp. *bulgaricus* IRL14-03 significantly reduced the recoverable CFUs of *Salmonella* in mice ($p \leq 0.05$). Among the three, *L. delbrueckii* subsp. *bulgaricus* IRL14-03 was able to decrease the viable number of *Salmonella* in the gut of mice compared with untreated *Salmonella*-infected control mice ($p \leq 0.05$). All three *Lactobacillus* isolates showed promising inhibitory activity against *Salmonella* in the mouse model, a cocktail of these isolates was tested on Cobb broiler chickens to determine its potential use as an antibiotic alternative. Daily treatment of poultry chickens with the *Lactobacillus* cocktail increased the recoverable CFUs of putative lactic acid bacteria (LAB) in the stool of chickens ($p \leq 0.05$). However, the putative LAB count decreased significantly ($p \leq 0.01$) upon induction of *Salmonella* infection. While there is a reduction of putative LAB numbers, the *Lactobacillus* cocktail can still mediate the reduction of *Salmonella* in chickens, especially during mid-stage farming ($p \leq 0.05$). Interestingly at this period, day 22 to day 28, the Cobb broiler chickens have reached the marketable size, comparable to the untreated and antibiotic-treated chickens. Therefore, animal productivity is maintained, as chickens are ready to be sold before day 35. The *Lactobacillus* isolates have comparable effects with the use of sub-therapeutic doses of antibiotics in preventing *Salmonella* infection and maintaining animal productivity of poultry chickens. But unlike antibiotics, the *Lactobacillus* cocktail does not form selective pressures in the environment that promote the development of antibiotic resistance. Hence, these isolates may be explored further to determine how they can be developed as an antibiotic alternative.

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INTRODUCTION

The poultry industry supplies approximately 36% of the global meat requirement. In 2016, it provided 120 million tons of poultry meat to keep up with the growing demands of the global market (FAO 2019). Concomitant with the high demands for food of animal origin is the shift towards more intensive food production systems that led to the increased use of antibiotics (FAO 2015). Poultry farmers use antibiotics in sub-therapeutic doses to prevent infections, maintain animal health, and promote growth (Philips et al. 2004). This has been practiced for many decades and is believed to help farmers increase their productivity (Ronquillo and Hernandez 2017). However, antibiotic use in agriculture is a double-edged sword. While it supports animal production, it also promotes the emergence of antibiotic-resistant microorganisms (Philips et al. 2004, Dibner and Richards 2005, Founou et al. 2016). The administration of low doses of antibiotics to animals selects for antibiotic-resistant strains over susceptible microorganisms resulting in a gut microflora dominated by antibiotic-resistant microorganisms (Apata 2009). Therefore, there is a need to explore alternative approaches that will minimize antibiotic usage in poultry farming while maintaining health and animal productivity.

Probiotics can be used as an alternative to antibiotics in poultry health management (Hossain et al. 2017). These microorganisms are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001, Hill et al. 2014). Probiotics are known to inhibit an array of pathogens (Collado et al. 2006, Leite et al. 2015) including those that cause gut infections such as *Salmonella* (Tellez et al. 2012). Among the well-studied probiotics are species of *Lactobacillus.* They produce lactic acid (Murry et al. 2004, Das et al. 2013), bacteriocin (Gong et al. 2010), and other compounds such as hydrogen peroxide (Pridmore et al. 2008), which have been reported to inhibit *Salmonella.* These *Lactobacillus* spp. have been used and tested for poultry management along with other probiotic species. By inhibiting the growth of *Salmonella,* lactobacilli can reduce the contamination of this pathogen from tissues of poultry animals (Wolfenden et al. 2007, Nouri et al. 2010, Carter et al. 2017). Many studies have supported the potential of *Lactobacillus* species as probiotics in the poultry industry and as an alternative for antibiotics use in agriculture (Murry et al. 2004, Mountzouris et al. 2010, Morgan 2017).

The benefits of *Lactobacillus*-based probiotics in agriculture are not limited to health management. Some studies demonstrated that probiotics could be used as growth promoters (Vicente et al. 2007, Vandeplas et al. 2009, Gadde et al. 2017). They can enhance the daily weight gain in poultry chickens comparable to antibiotics. Furthermore, they can decrease the feed conversion ratio, improve the nutrient digestibility coefficient, and modulate cecal microflora composition (Mountzouris et al. 2007, Gadde et al. 2017).

While probiotics offer a comparable efficacy as antibiotics in health management and animal productivity, it does not cause extensive selective pressures to the environment that could promote antibiotic resistance. Therefore, it is beneficial to capitalize on its potential in the poultry industry and agriculture. In this study, the potential of *Lactobacillus* spp. was evaluated in controlling *Salmonella* infection in Cobb broiler chickens. These were previously isolated and characterized (Nicdao et al. 2020) and found to inhibit *Salmonella in vitro* (MAC Nicdao and JA Ibana, unpublished report). The isolates were first tested on small animals, BALB/c mice, before proceeding to the experiment on poultry chickens. BALB/c mice were treated with the *Lactobacillus* spp. and infected with *Salmonella.* Recoverable cells of *Salmonella* were enumerated from the stool and the gut of mice. In the poultry chicken experiment, animals were infected with *Salmonella* while being administered with a cocktail of the three *Lactobacillus* isolates. The recoverable CFUs of *Salmonella* from chickens were counted and their weight gain was monitored to determine whether chickens treated with the cocktail have comparable weight with those treated with antibiotics. The results of this study thus present the ability of these *Lactobacillus* isolates to serve as an alternative to antibiotics in maintaining health and productivity in poultry farming.

MATERIALS AND METHODS

Microorganisms and culture conditions

Lactobacillus paracasei IRL14-01, *Lactobacillus casei* IRL14-02, and *Lactobacillus delbrueckii* subsp. *bulgaricus* IRL14-03 were previously isolated and characterized from commercial probiotic food products (Nicdao et al. 2020). The isolates are maintained in DeMan-Rogosa-Sharpe (MRS; Himedia, Mumbai, India) medium. The *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) was obtained from the Pathogen-Host-Environment Interactions Research Laboratory of the Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City, Philippines. The enteric bacterium is maintained in Trypticase Soy Medium (Himedia). The bacteria are stored at -80 ºC in 1:1 bacterial culture to 80% glycerol ratio (Zayed and Roos 2004).

Working cultures, used for treatments, were prepared from 24-hour cultures of bacteria incubated at 37 ºC. Each *Lactobacillus* working culture was adjusted to 1.00 X 10^9 CFU/mL cell concentration while the *Salmonella* working culture was adjusted to 1.00 X 10^8 CFU/mL. The working cultures were administered to mice or chicken according to the requirement of each treatment.

Animals, housing conditions, nutrition, and health management

Before the conduct of experiments, Institutional Animal Care and Use Committee (IACUC) certifications were acquired. One from the College of Science, University of the Philippines Diliman, Quezon City, Philippines, for the mouse model experiment and another from the College of Veterinary Medicine, Pampanga State Agricultural University, Magalang, Pampanga, Philippines, for the poultry chicken experiment.

Six to eight- week old BALB/c mice were obtained from the Marine Science Institute Animal Facility, College of Science, University of the Philippines, Diliman, Quezon City, Philippines. These were acclimatized for one week in standard polycarbonate cages, three mice per cage, at the animal house of the Institute of Biology, College of Science, University of the Philippines. Sterile bedding made from fine wood chips was provided and changed two times a week or when necessary. Sterile water and animal feeds were given *ad libitum.* A 12-hour light/12-hour dark cycle was maintained as well as a room condition of 23 ºC ± 2.0 ºC temperature and 40-60% humidity (The Jackson Laboratory 2016, Acurcio 2017).

Day-old Cobb broiler chicks were obtained from a local hatchery at Ayala, Magalang, Pampanga, Philippines. These were transferred to the animal facility of the College of Veterinary Medicine, Pampanga State Agricultural University. The chicks were grouped into six animals per cage made of PVC-coated wire mesh; each cage was separated by a galvanized steel sheet. Cages were designed to have removable

catch trays for animal litter, which were cleaned and disinfected every two days or when necessary.

Farming conditions and practices were adapted from undisclosed poultry farmers in Magalang, Pampanga to simulate actual poultry farming practices. Drinking water and animal feeds were given *ad libitum.* Feeds containing basic nutrients, devoid of antibiotics and probiotics, were requested and obtained from a local feed manufacturing company in Magalang, Pampanga, Philippines. All chicks were fed with booster feeds from day 2 to day 10, with starter feeds from day 11 to day 20, with grower feeds from day 21 to day 30 and with finisher feeds from day 31 onwards. The animals were also given freshwater containing vitamins and minerals during the day, and corresponding treatments at night. For the health management, animals were vaccinated for Newcastle disease on day 7 by ocular application and for infectious bronchitis on day 18 through their drinking water. The antibiotic administration practices of poultry farmers from Magalang, Pampanga were also adapted for the antibiotic-treated groups of chickens.

Evaluation of the inhibitory activity of *Lactobacillus* spp. against *Salmonella* Typhimurium in BALB/C mice

BALB/c white mice were grouped into four treatments with three mice each. Prior to the administration of treatments, all mice were screened for pre-exposure to *Salmonella.* Stool samples approximately 50 to 100 mg were collected, placed in sterile microcentrifuge tubes, and mixed with buffered peptone water (BPW; Himedia) in a 1:10 ratio. The tubes were incubated at 37 ºC for 18 ± 2 hours and a sample from each tube was transferred to Rappaport Vassiliadis Soy (RVS; Himedia) peptone broth and Tetrathionate broth (Himedia) for enrichment. The tubes were incubated at 42 ºC (RVS) / 37 ºC (Tetrathionate) for 20 ± 2 hours. After incubation, an inoculum from each enrichment tube was streaked separately onto *Salmonella-Shigella* agar (SSA; Himedia) and Xylosine Lysine Deoxycholate agar (XLD; Himedia) plates and incubated at 37 ºC for 24 hours (ISO 2007, ISO 2017, Schultz et al. 2018). All mice must not contain *Salmonella* to proceed to the experiment. The first group (T1, pre-/post-infection) was administered daily with 0.2mL of 1.0 x 10^9 CFU/mL working culture of *Lactobacillus* isolate (Hudault et al. 1997, Acurcio et al. 2017) for seven days using a gavage needle. After the 7-day *Lactobacillus* treatment, 0.2mL of 1.0 x 10^8 CFU/mL of *S.* Typhimurium (Hudault et al. 1997) was orally administered to the mice to induce gut infection. *Lactobacillus* treatment was continued for another 3 days. The second group of mice (T2, pre-infection) was administered with the same concentration of *Lactobacillus* for 7 days and infected with *Salmonella. Lactobacillus* treatment was ceased after infection. The third group of mice (T3, post-infection) was not given *Lactobacillus* treatment prior to induction of *Salmonella* infection but was subsequently treated with the *Lactobacillus* for three days with the same cell concentration. The fourth group was utilized as control and the mice were infected with *Salmonella* Typhimurium with no *Lactobacillus* treatment. Groups of mice in the experimental set-up had three sub treatments—*Lactobacillus paracasei* IRL14-01, *Lactobacillus casei* IRL14-02, and *Lactobacillus delbrueckii* subsp. *bulgaricus* IRL14-03.

On the fourth day of infection, stool samples were collected from each mouse. Mice were transferred one by one to a new disinfected cage and allowed to defecate until three to four pieces of stool were acquired from an individual mouse. The stool samples were placed in sterile test tubes using sterile forceps and processed for viable plate count of putative lactic acid bacteria and *Salmonella*. Afterward, all mice were sacrificed through cervical dislocation. The digestive tract (gut) including the liver, gall bladder, and spleen were collected and subjected to tissue processing for bacterial viable plate count the lactic acid bacteria and *Salmonella* cells that have colonized the digestive tract and that have invaded other organs were enumerated.

The stools collected from the mice were weighed and phosphate-buffered saline solution (PBS) was added to a ratio of 1 part stool and 9 parts PBS. The stool was homogenized and subjected to 10X serial dilution. One hundred microliters from each of the serially diluted tubes were inoculated onto a sterile plate and added with warm (40 ºC) sterile MRS agar (MRSA) for the pour plate technique. The MRSA plates were allowed to solidify, incubated for 48 hours at 37 ºC, and counted for colony-forming units (CFUs) of putative lactobacilli. On the other hand, one hundred microliters were also taken from serial dilution tubes and plated with SSA. The plates were also incubated for 24 hours at 37 ºC. The gut together with organ samples was macerated, placed in sterile tubes, weighed, and added with PBS solution to a 1:10 ratio. Processed gut samples were serially diluted and inoculum from each of the tubes was transferred onto MRSA and SSA plates as described previously. Plates containing 30-300 colony-forming units (CFUs) were used to compute for the standard plate count (Hudault et al. 1997, Maturin and Peeler 2001).

The carcasses were decontaminated using autoclave at 121 ºC for 45 minutes in 15 psi and disposed of through burying in a 3-feet deep pit.

Determination of the inhibitory activity of the cocktail of three *Lactobacillus* spp. against *Salmonella* Typhimurium in Cobb broiler chickens

Thirty-six newly hatched Cobb broiler chicks were grouped in six separate cages with 6 animals per cage. The first group (Atb, antibiotic control) was given alternately with sub-therapeutic doses of different antibiotics in their drinking water; administered three times per week. The second group (Lac, *Lactobacillus* control) was given a cocktail of three *Lactobacillus* isolates in their drinking water. The third group (Neg, untreated control) was left untreated and was given only with feeds and water. Groups 4, 5, and 6 were given the same treatments as groups 1, 2, and 3, respectively, and were infected with *Salmonella* Typhimurium (+Sal, *Salmonella*-infected groups). Similar to the mouse model experiment, chicks were screened for pre-exposure to *Salmonella* prior to the assay (ISO 2007, Marin and Lainez 2009, ISO 2017).

Upon transfer of the day-old chicks to their respective cages, the two antibiotic-treated groups (Groups 1 and 4) were given erythromycin for a day, then alternately with doxycycline, amoxicillin, and tetracycline on the succeeding weeks for three days a week, at a dose following manufacturer's recommendations for prophylaxis. Two *Lactobacillus*-treated groups (Groups 2 and 5) were administered with the *Lactobacillus* cocktail through oral gavage on day 1 and supplementation in their drinking water on the succeeding days until the last day of treatment. The remaining groups (Groups 3 and 6) were left untreated.

The chickens were infected with *Salmonella* Typhimurium using a dose of 1.00 X 10^4 CFU/mL (Higgins et al. 2010). The cells were administered through oral gavage on day 20 and day 35. Two days after infection (day 22 and day 37), the numbers of recoverable putative lactic acid bacteria and *Salmonella* from animals were determined by plating stool samples on MRSA and SSA. Fresh stool samples were collected from a sterile stool bag adhered to the anal region of animals. Each stool sample was diluted serially 10X in sterile PBS solution. One milliliter from each dilution tube was mixed separately with warm (40 ºC) MRSA and SSA, cooled, and incubated at 37 ºC. Colonies on SSA plates were enumerated 24 hours after incubation while co-

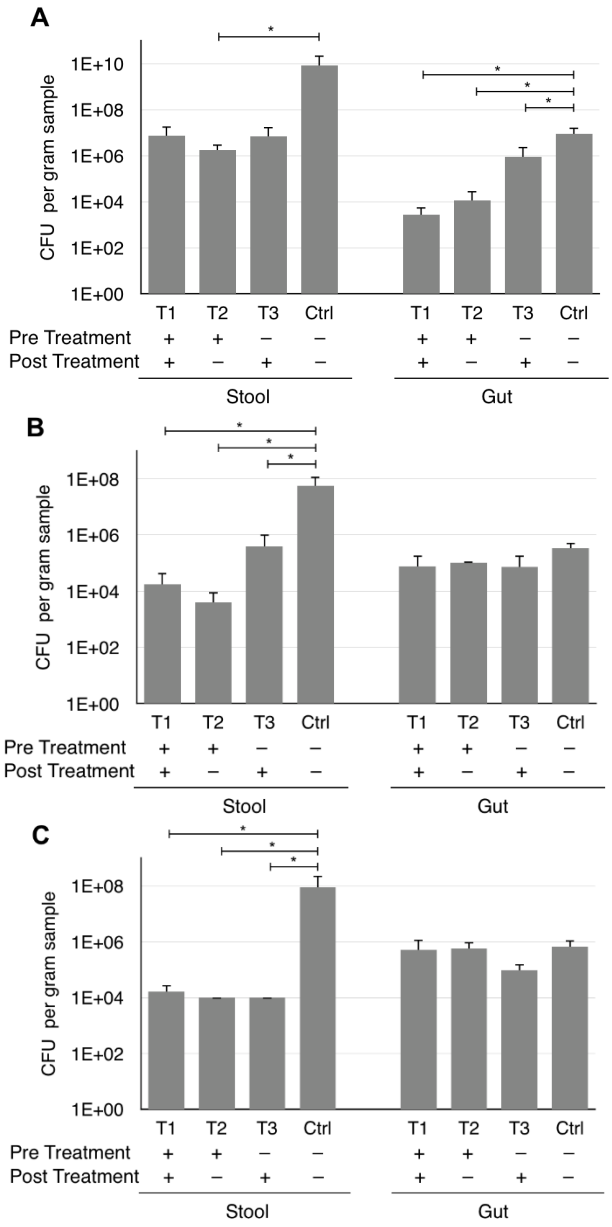

Figure 1: Recoverable CFUs of *S.* Typhimurium in the stool and gut of BALB/c mice treated with *L. delbrueckii* subsp. *bulgaricus* IRL 14-03 (A), *L. casei* IRL 14-02 (B), and *L. paracasei* IRL 14-01 (C). Oral administration of *Lactobacillus* isolates significantly reduced the *Salmonella* load in the stool of BALB/c mice. Significant reduction of *S.* Typhimurium in the gut was observed only in *L. bulgaricus*-pretreated mice. Mann-Whitney U-test was used to compare statistical significance of each treatment with the control. *$p \leq 0.05$

-lonies on MRSA plates were counted after 48 or 72 hours of incubation. Standard plate count was also used to compute for the recoverable CFUs (Hudault et al. 1997, Maturin and Peeler 2001).

Measurement of animal mass

The weight of each experimental animal was measured on day 1 and each week that followed, specifically, on days 7, 14, 21, 28, and 35. The average weight of each treatment group was computed from six animal replicates.

Statistical Analysis

All data collected were analyzed using IBM SPSS software version 25.0 with license number 3424. The normality test considered the data as non-parametric. Kruskal-Wallis one-way analysis of variance followed by pairwise comparisons using the Mann-Whitney U test was used to determine the statistical significance of each treatment to the negative treatment or control at p-value ≤ 0.05.

RESULTS

***Lactobacillus spp.* inhibit the growth of *S.* Typhimurium in the gut of BALB/c mice**

The *Lactobacillus* isolates inhibit *S.* Typhimurium *in vitro* (MAC Nicdao and JA Ibana, unpublished report). This *in vitro* observation was investigated how it is translated to the effect of isolates on pathogen clearance *in vivo*. Thus, BALB/c mice were exposed to different *Lactobacillus* spp. treatments, which included treatment prior (pre-treatment) to and after (post-treatment) infection (T1), pre-treatment only (T2), and post-treatment only (T3). The *S.* Typhimurium viable cells that can be recovered from the stool and the gut of treated mice were then compared to the recoverable *S.* Typhimurium in the untreated control.

It was observed that *Lactobacillus* treatment of mice using *L. delbrueckii* subsp. *bulgaricus* IRL14-03 significantly decreases the number of *Salmonella* in their stool (Figure 1A). In all treatment regimens, *L. delbrueckii* subsp. *bulgaricus* IRL14-03 was able to reduce the recoverable number of *S.* Typhimurium in stools by more than three logs. From the control *Salmonella* load of $8.58 \pm 14.83 \times 10^9$ CFU/gram of stool sample, the *Salmonella* recovered from stools was reduced to $7.70 \pm 11.44 \times 10^6$ CFU/gram in T1 ($p > 0.05$; ns), $1.80 \pm 1.31 \times 10^6$ CFU/ gram in T2 ($p \leq 0.05$), and $7.18 \pm 11.11 \times 10^6$ CFU/ gram in T3 ($p > 0.05$; ns). This *S.* Typhimurium load reduction was also observed in the gut of mice; from $8.84 \pm 7.41 \times 10^6$ CFU/gram of tissue sample in the control to $2.77 \pm 3.06 \times 10^3$ CFU/gram in T1 ($p \leq 0.05$), to $1.13 \pm 1.79 \times 10^4$ CFU/gram in T2 ($p \leq 0.05$), and to $9.14 \pm 14.79 \times 10^5$ CFU/gram in T3 ($p \leq 0.05$) (Figure 1.A).

When *L. casei* IRL14-02 was used to treat mice, it was only effective in reducing *S.* Typhimurium load in the stool. All treatment regimens using *L. casei* IRL14-02 significantly reduced the recoverable number of *Salmonella* in the stool by two to four logs (Figure 1.B). From an *S.* Typhimurium load of $5.76 \pm 5.98 \times 10^7$ CFU/gram of stool sample in untreated control, *Salmonella* was reduced to $1.73 \pm 2.83 \times 10^4$ CFU/gram ($p \leq 0.05$) upon *Lactobacillus* treatment prior and post-infection (T1), to $4.00 \pm 5.20 \times 10^3$ CFU/gram ($p \leq 0.05$) by *Lactobacillus* treatment pre-infection (T2), and $3.91 \pm 6.75 \times 10^5$ CFU/gram ($p \leq 0.05$) by *Lactobacillus* treatment post-infection (T3). However, *L. casei* IRL14-02 treatment failed to reduce the number of recoverable CFUs of *S.* Typhimurium in the gut of BALB/c mice (Figure 1.B).

Similar to *L. casei* IRL14-02, *L. paracasei* IRL14-01 was only able to decrease recoverable CFUs in the stool but not in the gut. Almost four-log decrease was observed from *S.* Typhimurium counts in the stool of all *L. paracasei* IRL14-01–treated mice regardless of the type of administration (Figure 1.C). From a *Salmonella* load of $8.91 \pm 13.93 \times 10^7$ CFU/gram of stool sample, *Lactobacillus* treatments reduced this to $1.67 \pm 1.15 \times 10^4$ CFU/gram ($p \leq 0.05$) in T1, and to $1.0 \pm 0.00 \times 10^4$ CFU/gram ($p \leq 0.05$) in both T2 and T3. In the gut, there was no statistical evidence showing changes in *Salmonella* load among treatments; counts ranged from 9.63×10^4 to 6.75×10^5 CFU/gram (Figure 1.C).

The results of the mice model experiment provided the needed data to test the potential of the three *Lactobacillus* isolates in controlling *Salmonella* infection in poultry farming. Because all isolates can reduce the recoverable CFUs of *Salmonella* in the stool of BALB/c mice, their anti-*Salmonella* activity as a

Lactobacillus cocktail was tested in a pilot set-up of Cobb broiler chickens.

***Lactobacillus* administration in drinking water increases the total recoverable putative lactic acid bacteria (LAB) from the stool of poultry chickens**

Livestock farmers in many countries still administer antibiotics to their animals to prevent infectious diseases and increase productivity (Katakweba et al. 2012, Van Boeckel et al. 2015, WHO 2017). However, rampant use of antibiotics in agriculture contributes to creating selective pressures in our environments that lead to the development of antibiotic-resistant microorganisms (Apata 2009, Landers et al. 2012, Katakweba et al. 2012, Van Boeckel et al. 2015). Therefore, the development of alternative approaches to reduce the use of antibiotics in poultry farming that can also control the growth of pathogens such as *Salmonella* is highly desirable. In this study, the testing of the anti-*Salmonella* activity of the *Lactobacillus* spp. was continued from the mice model to poultry chickens.

First, the administration of *Lactobacillus* cocktail was tested whether it can increase the total recoverable putative lactic acid bacteria (LAB) from the gut of chickens. A daily dose of a cocktail of *Lactobacillus* isolates was given to a group of experimental chickens for 37 days. Antibiotics were given to another group and the last group was left untreated. The total putative LAB CFUs in the stool of *Lactobacillus*-treated chickens (Lac) increased compared to the untreated control group (Neg) (Figure 2); from $5.00 \pm 5.68 \times 10^{10}$ CFU/gram of stool sample to $2.30 \pm 1.24 \times 10^{11}$ CFU/gram on day 22 ($p \leq 0.05$) and from $9.45 \pm 11.87 \times 10^{9}$ CFU/gram of stool sample to $1.05 \pm 1.10 \times 10^{11}$ CFU/gram on day 37 ($p > 0.05$; ns). Recoverable putative LAB counts in antibiotic-treated animals (Atb) were also lower compared to the control (Neg), but the differences were not statistically significant. Together, these data suggest that the *Lactobacillus* cocktail increases the total putative lactic acid bacterial population in the gut of poultry chickens.

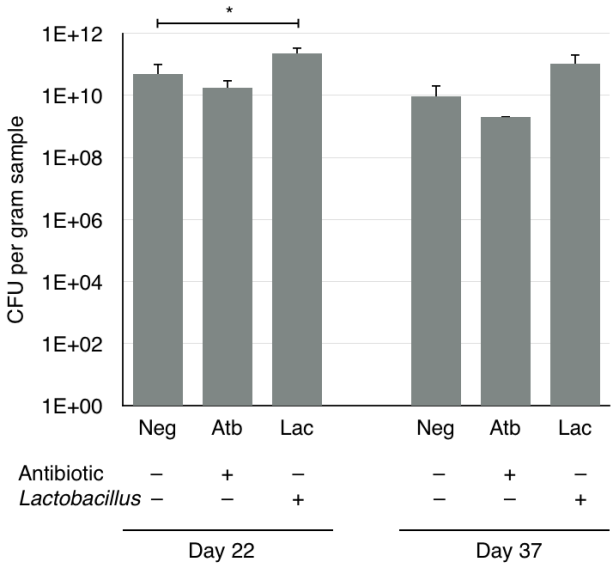

Figure 2: Viable counts of putative lactic acid bacteria in the stool of chickens at day 22 and day 37. Daily administration of a cocktail of *Lactobacillus* spp. to drinking water of poultry chickens (Lac) increased the recoverable putative lactic acid bacteria (LAB) in the stool of chickens compared with the untreated (Neg) and antibiotic-treated (Atb) set-ups (n=6). Data are representative of two independent experiments. A significant increase in recoverable putative LAB CFU was observed at Day 22 in *Lactobacillus*-treated animals (Lac) compared to the untreated control (Neg). Mann-Whitney U-test was used to compare statistical significance of each treatment with the untreated control. *$p \leq 0.05$

***Salmonella* infection reduces the total recoverable putative LAB from the stool of poultry chickens**

While the total recoverable putative LAB from the stool of chickens increases upon *Lactobacillus* administration, the effect of *Salmonella* infection on the putative LAB population was also determined. The total recoverable putative LAB counts were measured after *Salmonella* infection to find out if *Salmonella* counteracts the *Lactobacillus* treatment. Another group of chickens (Lac+S) administered with *Lactobacillus* was then infected with *Salmonella* on days 20 and 35. Counts revealed that the recoverable putative LABs were reduced significantly to $1.04 \pm 0.72 \times 10^{8}$ CFU/gram and $5.44 \pm 6.91 \times 10^{8}$ CFU/gram of stool samples on days 22 ($p \leq 0.01$) and 37 ($p \leq 0.01$), respectively, compared to *Lactobacillus* treatment alone (Lac) (Figure 3). These data suggest that *Salmonella* can antagonize the growth of LAB in poultry chickens. And that the frequency of administration or cell concentration of the cocktail should be increased to allow the *Lactobacillus* species to dominate the gut faster than *Salmonella* or other gut pathogens.

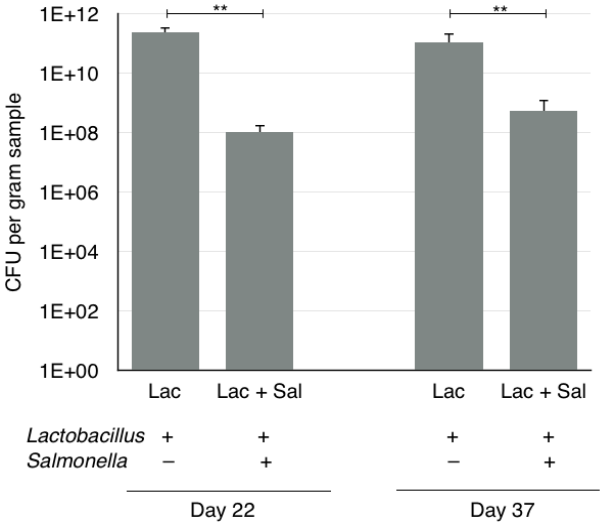

Figure 3: Viable counts of putative lactic acid bacteria in the stool of *Lactobacillus*-treated chickens after *Salmonella* infection. The recoverable CFUs of putative lactic acid bacteria in the stool of *Lactobacillus*-treated chickens (Lac+S) decreased significantly in chickens infected with *Salmonella*. Data were collected on day 22 and day 37. The graphs are representative of two experimental trials with six replicates per group (n=6). Mann-Whitney U test was performed to determine statistical significance between treatments. **$p \leq 0.01$

The cocktail of *Lactobacillus* spp. controls *Salmonella* infection in poultry chickens during mid-farming stage

To determine whether the cocktail of *Lactobacillus* spp. can control *Salmonella* growth in poultry chickens, the recoverable CFUs of *Salmonella* in the stool of different groups of *Salmonella*-infected chickens were assessed. The group of chickens (Atb+S) treated three times weekly with sub-therapeutic doses of antibiotics had a significantly lower number of *Salmonella* compared to the untreated group (Neg+S) (Figure 4). *Salmonella* CFU counts decreased from $1.64 \pm 1.46 \times 10^{5}$ CFU/gram of stool sample to $1.25 \pm 2.50 \times 10^{4}$ CFU/gram on day 22 ($p \leq 0.05$) and from $1.97 \pm 1.48 \times 10^{5}$ CFU/gram of stool sample to $3.33 \pm 2.94 \times 10^{3}$ CFU/gram on day 37 ($p \leq 0.05$). On the other hand, daily *Lactobacillus* treatment of chickens (Lac+S) reduced the recoverable CFUs of *Salmonella* in the stool on day 22 to $6.25 \pm 11.25 \times 10^{2}$ CFU/gram sample. *Salmonella* CFU counts were two-log lower compared to the untreated control—Neg+S ($p \leq 0.05$) and one-log lower compared to the antibiotic-treated group (Atb+S). Reduced counts of *Salmonella* were also observed at day 37, but differences with the untreated control were not statistically significant.

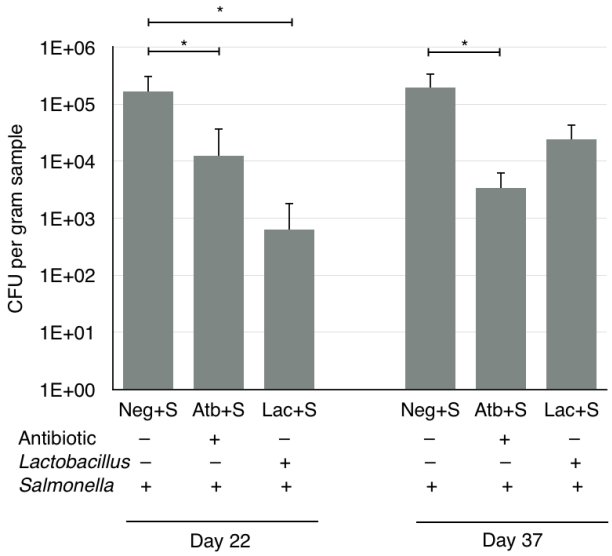

Figure 4: Viable counts of *Salmonella* in the stool of chickens on day 22 and day 37. Antibiotic and *Lactobacillus* treatments decrease *Salmonella* load in the stool of poultry chickens. While antibiotics (Atb+S) reduce significantly the pathogen load at day 22 and day 37, *Lactobacillus* treatment (Lac+S) effectively controls *Salmonella* infection on day 22. Data are representative of two trials of replicated samples (n=6). Statistical significance of treatments against the untreated control (Neg+S) analyzed using Mann-Whitney U test. *$p \leq 0.05$

The cocktail of *Lactobacillus* spp. maintains animal productivity in poultry farming; poultry chickens reach their marketable size before day 35

The data demonstrated that weekly treatment of poultry chickens with sub-therapeutic doses of antibiotics significantly reduces recoverable CFUs of *Salmonella* from stools of chickens on day 22 and day 37. Whereas, daily treatment with the *Lactobacillus* isolates significantly controls *Salmonella* infection at day 22, more potently than antibiotics.

Between days 22 and 37, which is the mid-farming period, chickens can already reach their marketable weight (Lastimoza 2006, Bernardo and Luis 2010). Therefore this study also examined the average mass of poultry chickens at different farming stages to determine if *Lactobacillus* treatment affects the weight of poultry chickens. Interestingly, results showed that all chickens—uninfected and *Salmonella*-infected—were nearly one kilogram (876±32 grams) on day 21. Among the uninfected groups, antibiotic-treated chickens (Atb) were significantly heavier than the untreated group (Neg) (Figure 5A, Day 21; $p \leq 0.05$), while all *Salmonella*-infected chickens had statistically similar average mass (Figure 5B, Day 21). On day 28, all chickens have a statistically similar mass, ranging from 1,215 to 1,360 grams with an overall average weight of 1,293 ±147 grams. At a later day (day 35), chickens have reached higher weights ranging from 1,766 to 1,955 grams.

Considering that some consumers prefer meats from younger or smaller size animals (Chang 2007, Bernardo and Luis 2010), poultry raisers may opt to market their chickens sooner than 35 days. Therefore, the data from this pilot study demonstrated that the *Lactobacillus* cocktail can be used as an alternative to antibiotics to maintain productivity and manage *Salmonella* infection when administered for harvesting poultry chickens at the mid-farming stage. By shifting from antibiotics to antibiotic alternatives, in this case, the *Lactobacillus* cocktail, the introduction of selective pressures in the poultry environment that promotes the development of antibiotic-resistant microorganisms can be prevented.

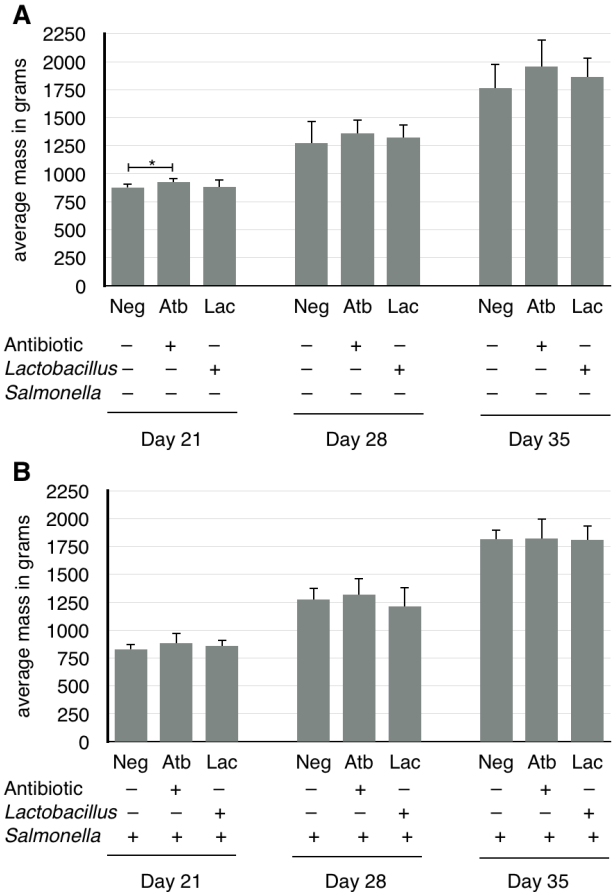

Figure 5: The average mass of chickens on day 21, day 28, and day 35. Both uninfected (A) and *Salmonella*-infected chickens (B) are nearly 1000 grams on day 21. Their weight is more than 1000 grams or 1 kilogram on day 28 and has reached a large size on day 35 weighing more than 1.5kg. Regardless of treatments (A and B), the chickens have statistically similar average mass on day 28 and 35 except on day 21, between antibiotic (A, Atb) and untreated uninfected chickens (A, Neg). Measurements are representative of replicated samples (n=6) from two trials. Statistical significance between samples was analyzed using the Mann-Whitney U test. *$p \leq 0.05$

DISCUSSION

This study examined the potential of a cocktail of *Lactobacillus* spp. in controlling *Salmonella* infection in Cobb broiler chickens. Initially, the results of the previous *in vitro* study on the anti-*Salmonella* activity of the *Lactobacillus* isolates (MAC Nicdao and JA Ibana, unpublished report) were validated by testing these isolates on an animal model using BALB/c mice. Interestingly, the *in vivo* assay collaborated with the previous results. The *Lactobacillus* isolates could also inhibit the growth of *Salmonella in vivo* as depicted by a reduced number of recoverable CFUs of *Salmonella* in stools of mice. Furthermore, an experiment was conducted on poultry chickens. Daily administration of a cocktail of the three isolates increased the recoverable putative LAB counts in the stool of chickens. Importantly, the *Lactobacillus* cocktail also significantly reduced the number of recoverable *Salmonella* in stools of chickens and achieved their marketable weights during the mid-farming stage.

Numerous species of *Lactobacillus* display inhibitory activities against enteropathogens such as *Salmonella*; they control pathogen growth through the production of 'soluble factors' and direct cell-to-cell interaction (Bermudez-Brito et al. 2012). These factors include bacteriocins (Tufail et al. 2011, Kumar et al. 2012, Wannun et al. 2014), lactic acid (Plessas et al. 2008,

Panesar et al. 2010, Nguyen et al. 2012), and other metabolites such hydrogen peroxide (Pridmore et al. 2008). The *Lactobacillus* cocktail likely contains one or some of these 'soluble factors'. In our *in vitro studies*, results have indicated that the three *Lactobacillus* isolates innately produce anti-*Salmonella* compound. Their cell-free culture supernatants significantly decrease the growth of *Salmonella* in a microbroth culture assay (MAC Nicdao and JA Ibana, unpublished report). Hence, when administered to poultry chickens, the *Lactobacillus* cocktail can mediate the reduction of *Salmonella* in the stool. We also cannot discount the possibility that the isolates may have interacted with the host cells and modulate the animal's immune response. Reports have described the ability of *Lactobacillus* species to mediate the immune response of poultry chickens to control *Salmonella* infection (Higgins et al. 2007, Yang et al. 2014, Penha Filho et al. 2015).

Social interaction among microorganisms is a natural phenomenon. It involves either competition or cooperation, but both play significant roles in microbial ecology, evolution, and infectious diseases (Li and Tian 2016). The results of the study demonstrate the competition between two types of bacteria in the gut environment of chickens—pathogenic *Salmonella* and commensal *Lactobacillus.* Like in most battles, both sides will take a beating. When the two find their way to the gut environment of chickens, they may proliferate and evolve into different phenotypes. Upon their interaction, the weaker strains of each species are killed and the more resilient phenotypes thrive (Ghoul and Mitri 2016). This study showed that daily administration of *Lactobacillus* spp. to chickens increases the number of putative LAB in the gut and may promote the proliferation of many strains. When infection was induced using *Salmonella*, the weak LAB strains may have been outcompeted by *Salmonella,* while the resilient LAB strains inhibited *Salmonella.* Therefore, decreased numbers of recoverable CFUs of putative LAB and *Salmonella* in the stool of chickens were observed. This observation may be useful in the actual poultry farming if the cocktail of *Lactobacillus* species will be used. Increasing the cell concentration or frequency of administering the cocktail to poultry chickens especially during the early period of farming may be performed. This could allow the *Lactobacillus* species to dominate the gut faster or better than *Salmonella* and possibly other gut pathogens. And because the first two weeks of animal growth are considered as a critical period for chicks, this is the best time to administer the cocktail of *Lactobacillus* species to work in concert with the immunological function and nutrient absorbing capacity of their gut (Friedman et al. 2003, Klasing et al. 2007, Cox and Dalloul 2015).

Selective forces driving bacterial competitions resulting in the survival of robust and more adaptive strains also depend on ecological conditions (Ghoul and Mitri 2016). The gut environment of the chickens may vary as chickens mature. The type of feeds given at specific age (Pan and Yu, 2014) and host factors such as age, sex, and breed (Wielen et al. 2002, Kers et al. 2018) may contribute to this variation. Booster feeds are given from day 1 to day 10, starter feeds from day 11 to day 20, while grower feeds are given from day 21 to day 30 and finisher feeds are given from day 31 onwards. These feeds vary in nutritional content especially in protein ratio and amino acid concentration (Bernardo and Luis 2010). It is hypothesized that the variation in the gut ecological condition of chickens may have affected the competitive interaction between *Salmonella* and *Lactobacillus* on day 20 and day 35. Although the *Lactobacillus* isolates can reduce CFU counts of *Salmonella* on day 22 and day 37, the ecological conditions of the gut during the mid-farming stage may have favored the LAB isolates compared during the later stage, thus, significantly reducing viable cells of *Salmonella* on day 22.

The importance of the findings was revealed as the average mass of the experimental chickens was examined. Data suggest that the size of Cobb broiler chickens treated either with antibiotics or *Lactobacillus* is not statistically different at day 21 and day 28 (Figure 5). Therefore, they can be collected for meat production before day 35. The weight of chickens at this farming stage, approximately 800 to 1,300 grams, is considered as marketable weight (Lastimoza 2006) because some consumers prefer small chickens (Bernardo and Luis 2010). The average masses of uninfected chickens on day 28 are 1,274 ±199 grams (Neg), 1,360 ±127 grams (Atb), and 1,323 ±119 grams (Lac). While for *Salmonella*-infected chickens, their average masses are 1,277 ±103 grams (Neg+Sal), 1,321 ±148 grams (Atb+Sal), and 1,215 ±172 grams (Lac+Sal). When these measurements are computed with hundreds or thousands of poultry animals against the farmgate price, the economic implications of using the cocktail of *Lactobacillus* may be derived. Therefore, a large-scale study may be designed to substantiate the use of this antibiotic alternative in poultry farming.

Studies have included *Lactobacillus* strains in broiler nutrition to boost animal growth, enhance feed conversion ratio, and improve nutrient apparent digestibility coefficient (Mountzouris et al. 2007, Mountzouris et al. 2010). They have suggested positive association of probiotics such species of *Lactobacillus* with poultry growth performance (Jin et al. 1996, Vuong et al. 2016, Wang et al. 2017) and with the maintenance of animal health especially in the prevention of *Salmonella* infection (Pascual et al. 1999, Foltz et al. 2017, Nakphaichit et al. 2019). To my knowledge, there are no reports of *Lactobacillus* species controlling *Salmonella* in chickens while promoting growth performance. Studies are focused either on controlling *Salmonella* infection of poultry chickens (Vicente et al. 2008, Tellez et al 2012, Nakphaichit et al 2019) or on enhancing animal productivity in poultry farming (Gunal et al. 2006, Murshed and Abudabos 2015, Chen et al. 2017). This study, on the other hand, showed that the *Lactobacillus* cocktail has anti-*Salmonella* activity on day 22 or during the mid-farming stage while maintaining animal productivity in poultry farming.

Therefore, this study presented the feasibility of using these *Lactobacillus* spp. isolates as an alternative approach to antibiotic usage in poultry farming. The use of these isolates can maintain productivity and control gut infections caused by *Salmonella* while reducing the selective pressures that drive the emergence of antibiotic-resistant microorganisms (AMR). With the alarming contribution of agricultural industries to AMR, it is recommended that studies on farming protocols using antibiotic alternatives in poultry farming and other livestock animals be pursued further. Also, our *Lactobacillus* isolates must be fully characterized to reveal their potential as probiotics and possible use in agriculture.

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CONFLICT OF INTEREST

The author declares 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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