

## COMPARISON OF NOVEL *BACILLUS SALMALAYA* 139SI AND *LACTOBACILLUS AS* PROBIOTICS IN THE DRINKING WATER OF CHICKS

Efrizal<sup>1,2</sup>, S. Ismail\*<sup>1</sup>, A. A. Ajeng<sup>1</sup>, M. R. Ramli<sup>1</sup>, F. Ameen<sup>3</sup>, N. Nazirah Md. Nasir<sup>1</sup> and M. Lakshmikandan<sup>4</sup>

<sup>1</sup> Institute of Biological Sciences, Faculty of Science, Universiti Malaya 50603, Kuala Lumpur, Malaysia.

<sup>2</sup> Faculty of Agriculture, Jambi University, Jambi-Sumatera 36657, Indonesia.

<sup>3</sup> Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia

<sup>4</sup> College of natural resources and environment, Northwest A&F University, Yangling Shaanxi China

Corresponding author's email: [salmah\\_r@um.edu.my](mailto:salmah_r@um.edu.my)

### ABSTRACT

Salmonellosis is a commonly occurring bacterial disease in chickens that leads to increased mortality, as well as a decline in the quality and quantity of hen eggs. This, in turn, causes financial losses in the poultry industry. The objective of this research was to investigate the potential of *Lactobacillus plantarum* and novel *B. salmalaya* 139SI as probiotics in to improve performance of laying hens and the quality of eggs. The study at Chuan Chuan Poultry Farm employed a completely randomized design, rearing 30,000 Hisex brown layer day-old chicks in three groups—control (antibiotics only), *B. salmalaya* 139SI probiotic, and *L. plantarum* probiotic—over 10 months, with probiotics administered in drinking water at a 0.5% concentration. Variables observed include growth performance of layer, egg weight, the different components of the eggs, such as the eggshells, egg yolks, and egg whites, were also examined to detect the presence of *Salmonella* spp. Various tests were conducted, including biochemical testing, serotyping, conventional detection using specific agars, and a modern detection method utilizing 16S rRNA sequencing. *L. plantarum* treatment in drinking water showed significantly different results ( $P < 0.05$ ) than the others, with a final bodyweight of  $2071.98 \pm 41.72$  g/chick/10 months and egg weight of  $69.52 \pm 2.36$  g/egg. The results from all the tests indicated that both probiotics, *L. plantarum* and *B. salmalaya* 139SI, can produce eggs free from *Salmonella* contamination. Moreover, the treatment with *L. plantarum* demonstrated significant protective effects against *Salmonella* infection in the eggs, surpassing the protective capacity of *B. salmalaya* 139SI.

**Keywords:** Salmonellosis, probiotic, *Salmonella*-free eggs,

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### INTRODUCTION

Numerous nations have always relied heavily on the livestock sector, particularly the poultry business. Economic losses in the poultry sector are frequently linked to deteriorating environmental conditions, avian stress exposure, and the spread of disease. Salmonellosis is one of the most prevalent illnesses in livestock. Salmonellosis is an enteric illness that is brought on by a *S. enteritidis* bacterium infection. The poultry business is very concerned about this zoonosis because it causes decreased in productivity, higher mortality rates, and contamination of poultry products intended for human consumption (Patterson and Burkholder, 2003). Probiotics have been successfully used as supplements for poultry feeding and replacement of antibiotics as biological agents in the prevention of pathogenic bacteria infestation. Probiotics generally refer to bacterial cultures that are able to stimulate the gut microflora and modulate the environment of the digestive tract in a positive way,

resulting in improved growth performance of chicken and efficiency of chicken feed (Jeong and Kim, 2014).

Eggs are sporadically infected by *Salmonella* spp. from both on the outside and inside. Internal contamination can occur as a result of penetration through the eggshell or by direct contamination of egg contents prior to oviposition caused by infection of the reproductive organs. Once within the egg, the pathogen must deal with antimicrobial substances in the albumen and vitelline membrane before migrating to the yolk (Gantois *et al.*, 2009). In response to intestinal pathogens, probiotics produce antibiotic-like chemicals, such as lactic and acetic acid, and they compete with other organisms for nutrition and adhesion sites. In addition, there are changes in the amount of antibodies, macrophage activity, and enzyme activity (Hose and Sozzi, 1991). Avila *et al.* (2006) observed a greater reduction of infections in probiotics administered chicks, indicating the relevance of early probiotic usage in infection prevention.

The use of antibiotics in poultry farming to promote development and prevent diseases offers substantial issues, including the creation of antibiotic-resistant bacteria, residues in chicken products, and environmental pollution (Muaz *et al.*, 2018). These concerns have resulted in regulatory limits and a rising need for alternatives. Probiotics, which are living microorganisms that provide health advantages, have emerged as viable alternatives. Probiotics have a favorable impact on poultry intestinal health, prevent infections without contributing to resistance, boost growth performance, and are ecologically friendly (Alagawany *et al.*, 2018; Jha *et al.*, 2020; Ng *et al.*, 2024). Although promising, further study is needed to improve their application and understand particular mechanisms, assuring their efficacy as sustainable alternatives in chicken production (Haque *et al.*, 2010; Yang *et al.*, 2015).

To the best of our knowledge, no research on *Salmonellosis* inhibition in chicken layers eggs has been conducted that focuses on early administration of probiotics in the feeding regime of newly hatched chick layers. A chick's first 21 days of life are crucial for the development and maturation of the bird's microbiota. The gut microbiota changes gradually throughout this time, with colonization starting soon after hatching (Valenzuela, 2021). As a result, administering probiotics from an early age till egg production in later developmental stage may give a comprehensive knowledge of the effect of probiotics in *Salmonella* inhibition in the commercial chicken farming business. In addition to *Lactobacillus*, *Bacillus* is another probiotic that is frequently employed in the livestock business. A Gram-positive bacterium called *Bacillus* possesses endospores that are extremely resistant to environmental factors including severe pH and low/high temperatures (Ajeng *et al.*, 2023; Hung *et al.*, 2012). *Bacillus cereus*, *Bacillus clausii*, and *Bacillus pumilus* are among the five commercial probiotic products consisting of bacterial spores that have been characterized and have the potential for their colonization, immunostimulation, and antimicrobial activity (Ajeng, 2021; Duc *et al.*, 2004). Even though probiotics have been shown to have positive effects in numerous studies, research demonstrates that the various reactions observed are still impacted by the probiotic strains, storage conditions, mode of administration (feed or water), dosage, feed mix, environment, and duration of treatment (Adebisi *et al.*, 2012). The objective of this research was to investigate the potential of *Lactobacillus plantarum* and novel *B. salmalaya* 139SI as probiotics in to improve performance of laying hens and the quality of eggs.

## MATERIALS AND METHODS

**Prebiotic preparation:** *Lactobacillus* bacteria were isolated from buffalo milk obtained from a local market in Kerinci, Jambi, Sumatra Indonesia and identification of the bacteria was carried out according to the procedure described by Sambrook *et al.*, (2006). The *B. salmalaya* 139SI isolate used in this study is a new bacterial strain isolate (Accession No. JF825470) which is a collection from UMMBTL, Institute of Biological Sciences, Faculty of Science, Universiti Malaya was previously isolated by our research team from agricultural soil (Ismail *et al.*, 2012). *L. plantarum* was culture using a medium containing 52 g/L of MRS broth, while the medium for *B. salmalaya* 139SI contained 37 g/L of BHI broth ( $1 \times 10^{12}$  CFU/mL). The inoculum was inoculated aseptically in sterile medium at a ratio of 1:10 and incubated for 48 hours at 37 °C.

**Experimental design:** The studies have been approved by the Ethics Committee of the University of Technology MARA (UiTM) Puncak Alam Campus Selangor and by the guidelines for the care and use of animals. The study was carried out at Chuan Chuan Poultry Farm, No. 1034, Jalan Telok Mengkuang, 42500 Telok Panglima Garang, Selangor. The experimental design in this study used a completely randomized design with subsamples (Hinkelmann and Kempthorne, 2007) by rearing 30,000 Hisex brown layer DOC (Hisex.com, The Netherlands-EU) for 10 months until egg laying. Chicks were divided into three groups and each group consist of 10,000 chickens (132 replicates, and each replicate consist of 8 chickens). First group (A) were control group that received antibiotic only. Second group (B) were chickens received probiotic *B. salmalaya* 139SI in drinking water and another group (C) were chicken received *L. plantarum* in drinking water. The chicken reared in a closed house system that implemented proper biosecurity. Chickens were fed and watered ad libitum. The route of probiotic administration was via drinking water with konsentration 0.5% of *B. salmalaya* 139SI or *L. plantarum*, respectively. Control groups only received antibiotics. The probiotics drinking formula were given to day old chicks until they are 10 months old. Parameters determined in the trial groups were compared with parameters determined in the control group. The batch media used for *L. plantarum* comprised 52 grams/liter of MRS broth, whereas the *B. salmalaya* 139SI media utilized contained 37 grams/liter of BHI broth. The starter culture inoculum was aseptically injected in sterile batch medium in a 1:10 proportion and incubated for 48 hours at 37 °C, with the inoculum predicted to contain  $10^{12}$  CFU/ml at the conclusion of the incubation and was utilized as a batch culture (probiotics). Probiotics were maintained at room temperature until use, and for long-term storage, they were kept in an area that was frigid.

The linear model used in this experiment is shown in Equation 1.

$$Y_{ijk} = \mu + \tau_i \epsilon_{ij} + \delta_{ijk} \quad (\text{Eq.1})$$

Where:

$Y_{ijk}$  = the value of observation in this treatment of  $i$ , in a retrieval to  $j$  and on the sample unit to  $k$ .

$\mu$  = common average value,

$\tau_i$  = the effect of the treatment to  $i$ ,

$\epsilon_{ij}$  = the trial error of treatment to  $i$  and repeat to  $j$ ,  $\delta_{ijk}$  = component error sample.

Parameters observed include such as: (i) body weight, and (ii) Egg weight, obtained from weighing chicken eggs every month and carried out from the twentieth to forty weeks.

### Observed variables

**Growth performance of layer and Egg Weight:** The assessments encompassed cage-side observation, individual monitoring for mortality, signs of illness, injury, or abnormal behavior, conducted once within the initial 30 minutes post-dosing and periodically during the

initial 48 hours (with emphasis on the first 4 hours), followed by daily observations for 14 days. Individual weights were recorded on Day 7 and Day 14 (termination) post-dosing, and all animals underwent sacrifice on Day 14, with subsequent gross necropsies. Egg weight, obtained from weighing chicken egg every month and was carried out from the twentieth to the forty weeks (Scott *et al.*, 1982).

### Evaluation of the *S. enteritidis* -free and infected eggs:

Eggs were gathered and examined for *S. enteritidis*, targeting eggshells, egg white, and yolk sacs. Detection methods encompassed both conventional and molecular analyses utilizing 16S rRNA. Conventional techniques involved sample inoculation on selective media, encompassing pre-enrichment and enrichment, followed by biochemical and serological testing. Molecular analysis employed DNA amplification techniques using enzymes with specific size through temperature-induced mechanisms (Sambrook *et al.*, 1989).

**Table 1. Types of probiotics used in this study.**

| Group        | Types of probiotics            | Total No. of chicken layers | *No. of random sampling per selection for each egg's parameter (8 chickens sampling of each 132 cages/group) |
|--------------|--------------------------------|-----------------------------|--|
| A            | Control (antibiotic)           | 10,000                      | 1,056  |
| B            | 0.5% <i>B. salmalaya</i> 139SI | 10,000                      | 1,056  |
| C            | 0.5% <i>L. plantarum</i>       | 10,000                      | 1,056  |
| <b>Total</b> |                                | 30,000                      | 3,168  |

### Conventional Detection Methods for *S. enteritidis* in Eggs Samples

**Selective Medium Tests:** The eggs were collected from the farm suspected or known to have *Salmonella* and sent for bacteriological examination. Eggs of farm pooled, and each treatment consisted of 20 eggs taken at random. Eggshells were cleaned and drained with a vigorous brush. The eggs were immersed in a solution of 200 ppm chlorine containing 0.1% sodium dodecyl sulphate (SDS, Merck Germany) for 30 min and air-dried at room temperature for 10 min (Andrews *et al.*, 2007). 25 g of eggshell samples (eggshells, egg whites and egg yolks) were aseptically weighed and placed into 225 mL lactose broth as a pre-enrichment medium, homogenized for 1-2 min and incubated at 35°C for 24 h ± 2 h. Then, 10 mL of pre-enrichment culture was added into 100 mL of tetrathionate brilliant green broth as enrichment media and incubated at 43°C ± 2°C for 24 ± 2 h. One loop of enrichment culture was streaked onto a petri dish containing selective media consisting of Hektoen enteric agar (HEA), Brilliant Green Agar (BGA) and MacConkey, and incubated at 35°C for 24 h ± 2 h. The colonies which were suspected of *Salmonella* spp. were streaked on Nutrient agar (NA) and incubated at 35°C for 24 ± 2 h and semi-solid Nutrient Agar (NA) for further serotyping (Andrews *et al.*, 2007). The colonies were

subsequently streaked into biochemical test media such as Lysine iron agar, Urea Agar, and Triple Sugar Iron Agar before incubated at 35°C for 24 h ± 2 h (Assays were conducted in triplicates).

**Serotyping of *S. enteritidis* :** The serotyping principle to identify heat-resistant somatic O antigens was performed by the suspension of bacterial cells of *Salmonella* spp. heated to 100°C for 1 h before being subjected to antiserum B, C, D, and E. As for the identification of the known *Salmonella* H antigen, it was performed by growing the *Salmonella* bacterium in semisolid media with a Craigie tube. A suspension of living bacterial cells made from cells outside the Craigie tube was treated with anti-H antiserum specific antibodies of poly Ha, Hb, Hc, Hd, He and H polyz. Then, the positive poly H group was broken down into the group H antigen numbers. Samples were expressed positive for *S. enteritidis* when they came from group D, identifications O: 1, 9, 12, and H: g, m, 1 and 7 according to the Kauffman-White scheme (Grimont and Weill, 2007).

**Determination of Antigen 'O':** Determination of antigens O or somatic was performed by using antiserum polyvalent or monovalent of groups B, C, D and E following procedure outlined by DebRoy *et al.* (2011) with slight modification as this group is commonly found

in animals (Frydendahl, 2002; Kamal *et al.*, 2019). If one of the groups of antisera is positive, then test is performed with the following appropriate antisera factor: (i) Group B, using single factors 4, 5, 12, 27, (4 and 5) are group B specific antigens; (ii) Group C, using single factors: 6.7; 6.8; 14.8 (20); 16.4; (iii) Group D, using single factor serum 12,46. Group D has a specific factor of 9. The somatic antigen 9 is a diagnostic factor for group D and it can be combined with factors 12 and 46; and (iv) Group E, using single factor serum 10, 15, 19. When agglutination with a factor of 15, it is then tested with a factor of 34 and subsequently adjusted to the Kauffman-White scheme (Andrews *et al.*, 2007).

**Determination of the serotype associated with the H antigen:** Bacterial suspension of *Salmonella* spp. used was the same as the bacterial suspension for the antigen O test, then tested against antisera polyvalent H: Ha, Hb, Hc, He, g complex and poly Z. Bacteria were grown in semisolid media with Graigie tubes and then scratched in the tube and then incubated at 37°C for 24 h. Bacterial suspensions to be used for antigen H testing were taken from outside the Graigie tube on a semi-solid surface. In the event of reaction with one of the antisera above, the bacteria was then tested with one of the following antisera: (i) H: a, b, c, d, I; (ii) Hb: K, lu/lw, r, b, z; (iii) Hc: 2, 5, 6, 7; (iv) He: h, x, Z15; (v) Hg: ge, f, m, s, t, p, g, u; and (vi) Poly z: z6, z10, z4, z23, z29, z38. In the absence of agglutination with the above antisera (H), hanging drop preparations, to determine the motility of the bacteria being tested. If the bacteria are motile, the bacteria culture were grown in the differentiation media, and a single colony was then taken for complete biochemical testing to determine if the bacteria was actually *Salmonella* spp. or otherwise. When true, a *Salmonella* spp. test was performed to determine the serotype. After determination/testing of the O and H antigens was completed, results were validated and matched to the antigenic structure obtained with the Kauffman-White scheme to obtain the name of the serotype *Salmonella* spp. tested (Andrews *et al.*, 2007).

***Salmonella* spp. detection method in eggs samples using PCR:** On the isolates, the 16S rRNA molecular DNA sequencing method was used (Herman, 2004; Sambrook *et al.*, 2006) for evaluating the *S. enteritidis*-free eggs by using *S. enteritidis* code number 4301/15 as a positive control. Eggshell disinfection and preparations, egg yolk, and egg white were carried out as described in following sections:

**Genomic DNA extraction, visualization, and amplification from eggs samples:** The eggshell, egg white and egg yolk genome tissue genome DNA were extracted using the GeneAll® Exgene™ kit (GeneAll Biotechnology co, Ltd.-Cambio-United Kingdom) according to the manufacturer's instructions. Mini

Horizontal Gel Electrophoresis (USA) was used to validate isolated genomic DNA. The standard protocol was followed (Voytas, 2000). The DNA fragments were visualized by staining the gel with fluorescent red gel staining dye for 5 to 10 min and then washed with aquades for 10 min. Gel was held at 4°C while bands on it were seen using UV transilluminators.

**Gene amplification:** The amplification processes were carried out using PCR Cycler Thermal Biosystem Verity 96 PCR machine (Fisher Scientific, USA). S212-Forward (5'AAACGTTTATCGTTACGCCG-3') and S6-Reverse (5' GTCAATGCGCCGTAATCATT-3') 16S rRNA specific primers were utilized to amplify the 16S rRNA region (Drahovská *et al.*, 2001). The reaction was performed in a total volume of 25 uL with a final concentration of 1X, consisting of 13.75 L of Water nuclease-free, 5.0 L of 5x PCR Buffer, 0.5 L of dNTP mix (10 mM), 1.25 L of Forward primer (10 uM), 1.25 L of Reverse primer (10 M), 3.0 L of DNA Template, and 0.25 L of Thermostable DNA polymerase (2 U/L). All of the mixture's components were put in a thin-walled PCR tube and vortexed briefly with a microcentrifuge. A PCR machine was then used to amplify the mixture following the manufacturer's protocol. Contig assembly was used to create the whole 16S rRNA DNA sequence by VectorNTi (Invitrogen, USA), and the BLASTN tool was used to compare it to other DNA sequences in the Genbank database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

**Data collection and analysis:** All data was collected and recorded for further analysis. Statistical data evaluation was provided by the program Microsoft excel. Differences in the calculation data between treatment and control were compared with analysis of variance (ANOVA) at the 95% significance level and if significantly different ( $P < 0.05$ ) Duncan tests were performed using the IBM SPSS 25 statistical program.

## RESULTS AND DISCUSSION

**Growth Performance of Laying Hens and Eggs Properties as Influenced by Early Probiotics Administration in Drinking Water:** The study investigated the relationship between probiotic administration and observed parameters, including layer performance and egg weight. The experimental design involved three groups: a control group receiving antibiotics only, a group receiving *B. salmalaya* 139SI probiotic in drinking water, and a group receiving *L. plantarum* probiotic in drinking water. The performance of layers and egg weights were monitored over a 10-month period, with probiotics administered at a concentration of 0.5%. The assessment included cage-side observations, body weight measurements, and pathology examinations, providing a comprehensive

analysis of the impact of probiotics on the specified parameters.

Table 2 and Table 3 showed the effects of probiotics supplementation in drinking water on chicken body weight, and egg weight, respectively during the feeding phase, respectively. In regards of the chicks' layer body weights in the trial,

the average beginning bodyweight was  $60.0 \pm 0.0$  g/chick. *L. plantarum* subsampling 3 (C3) showed significantly different results ( $P < 0.05$ ) than the others, with a final bodyweight of  $2071.98 \pm 41.72a$  g/chick/10 months. and egg weight of  $69.52 \pm 2.36$  g/egg. Meanwhile, for group B treatment with *B. salmalaya* 139SI, the average body weight of chicks recorded were  $1947.39 \pm 38.92bc$  g/chick/10 months, and egg weight was  $66.27 \pm 2.20ab$  g/egg. *L. plantarum* demonstrated the greatest increase in bodyweight growth in the first and second months following probiotic administration, followed by *B. salmalaya* 139SI treatment and control. The control treatment had the biggest bodyweight gain in the third and fourth months following probiotic administration, followed by the *B. salmalaya* 139SI and *L. plantarum* treatments. The treatment with *B. salmalaya* 139SI demonstrated the greatest bodyweight growth in the fifth and sixth months following probiotic administration, followed by the treatment with *L. plantarum* and the control. From the seventh to the tenth months, the *L. plantarum* treatment exhibited the greatest rise in bodyweight growth, followed by the *B. salmalaya* 139SI therapy and the control. These observations suggest temporal variations in the effects of the different probiotic treatments on bodyweight gain in the study subjects (Liu *et al.*, 2016; Park *et al.*, 2013).

Based on the chicken egg weight graphs gain during the probiotic drink period, *Lactobacillus plantarum* subsampling 3 (C3) showed significantly highest egg weight compared to the other treatment groups. This interesting finding highlights the possible role of *Lactobacillus plantarum* in increasing egg weight in chicken. This observation is significant in the context of chicken production because it indicates a practical and real advantage of adding this specific probiotic strain. The statistical significance not only validates the observed benefit, but also encourages further investigation into the processes by which *Lactobacillus plantarum* may positively affect egg weight. This crucial result necessitates further evaluation of the larger implications for chicken farming techniques and opens the door to further focused study to maximize the use of probiotics in boosting egg production efficiency with the probiotics. The overall administration of *Lactobacillus plantarum* probiotics among them subsampling 3 (C3), 2 (C2) and 1 (C1) showed the highest increase in egg weight gain in laying hens compared to the *B. salmalaya* 139SI and control during the experimental period. *Lactobacillus plantarum* increased egg weight in this

experiment was also higher than the standard of Hisex brown strain (Hisex.com, The Netherlands-EU). The *Lactobacillus* treatment produced findings that are similar to those observed by (Tortuero and Fernandez, 1995) in that the use of vital biomass of probiotic supplementation significantly influenced the weight of chicken eggs ( $P < 0.05$ ). However, some researchers reported that the inclusion of *Lactobacillus* or the addition of biological additives to laying hens that consume probiotics did not significantly affect the quality and weight of eggs (Aghaii *et al.*, 2010; Getachew *et al.*, 2016; Lei *et al.*, 2013). The exact methods by which these probiotic strains interact with the human immune system are also worth investigating. *L. plantarum* and *B. salmalaya* 139SI may trigger distinct immunological responses in layer chicks, altering their ability to withstand infections and stresses and, as a result, changing growth rates and egg quality. Furthermore, the ability of various probiotic strains to cling to intestinal epithelial cells and compete for resources with pathogenic microbes may play a role in their variable effects on chicken health. Furthermore, changes in the synthesis of beneficial metabolites by *L. plantarum* and *B. salmalaya* 139SI, such as vitamins and enzymes, may lead to variances in nutrient availability and utilization. These variances may have an impact on the overall efficiency of nutrient absorption, altering growth metrics and egg quality.

The favorable influence of *L. plantarum* and *B. salmalaya* 139SI on chicken development and egg quality has potential implications for the economic landscape of poultry farming, according to the research. The considerable increase in egg weight linked with *L. plantarum*, namely subsampling 3 (C3), indicates a possible path for enhancing egg production efficiency. Larger eggs frequently attract higher market prices, thus benefiting chicken farmers (Hilimire, 2012). Furthermore, better development rates in hens impacted by these probiotic strains might lead to faster turnover and shorter time to market, resulting in economic benefits for poultry producers (Abd El-Hack *et al.*, 2020; Ramlucken *et al.*, 2020).

#### Detection of Salmonella in the eggs from laying hens administered with different probiotic types.

**Conventional detection method:** The findings from conventional detection methods employing Hektoen Enteric Agar (HEA), Brilliant Green Agar (BGA), and MacConkey are illustrated in **Figure 1**. Biochemical tests were conducted on isolates suspected of *Salmonella* spp., involving Triple Sugar Iron Agar (TSIA), Lysine Iron Agar (LIA), and Urea Agar. The detailed test results can be found in **Table 5**. In summary, the examination of eggs, encompassing eggshell, egg white, and egg yolks from each treatment, revealed negative results, indicating the absence of *Salmonella* spp. HEA media functions as both a selective and differential agar medium.

Table 2: Effects of probiotics on chicken body weight.

| Treatments | Body weight (g/chick/month) |                 |                   |                  |                  |                   |                   |                   |                   |                   |
|------------|-----------------------------|-----------------|-------------------|------------------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|            | M1                          | M2              | M3                | M4               | M5               | M6                | M7                | M8                | M9                | M10               |
| A          | 204.83 ± 4.18b              | 591.49 ± 16.15a | 1032.99 ± 26.39a  | 1486.10 ± 42.14a | 1523.16 ± 40.96a | 1809.45 ± 45.54b  | 1832.24 ± 37.55b  | 1883.86 ± 19.79bc | 1919.63 ± 34.74bc | 2001.40 ± 17.44b  |
| B          | 227.37 ± 8.96ab             | 504.59 ± 11.75b | 1025.05 ± 37.52ab | 1376.30 ± 49.38b | 1552.32 ± 51.70a | 1846.52 ± 50.48a  | 1913.26 ± 50.58ab | 1919.11 ± 50.91b  | 1926.14 ± 50.21b  | 1947.39 ± 38.92bc |
| C          | 237.96 ± 9.31a              | 513.86 ± 17.23b | 951.20 ± 34.76b   | 1316.44 ± 35.51b | 1528.58 ± 54.87a | 1836.31 ± 43.12ab | 1932.46 ± 81.21a  | 2041.21 ± 43.41a  | 2057.94 ± 44.26a  | 2071.82 ± 41.72a  |

**Description:** A: Control, A1, A2, and A3: Subsampling; B: *B. salmalaya* 139SI, B1, B2, and B3: Subsampling and C: *L. plantarum*, C1, C2, and C3: Subsampling. Values are mean ± standard deviations, values with the different superscript alphabet in the same column show significantly different results ( $P < 0.05$ ).

Table 3: Effects of giving probiotic drinks on chicken egg weight.

| Treatment | Egg weight (g/egg/month) |                |                |                |
|-----------|--------------------------|----------------|----------------|----------------|
|           | M5                       | M6             | M7             | M8             |
| A         | 36.78 ± 3.37c            | 39.23 ± 4.83c  | 52.02 ± 2.54b  | 57.95 ± 3.45c  |
| B         | 52.48 ± 5.31ab           | 58.16 ± 2.32ab | 61.88 ± 3.01ab | 64.21 ± 1.43ab |
| C         | 55.18 ± 2.18a            | 61.96 ± 3.05a  | 64.70 ± 3.46a  | 66.45 ± 3.46a  |

**Description:** A: Control, A1, A2, and A3: Subsampling; B: *B. salmalaya* 139SI, B1, B2, and B3: Subsampling and C: *Lactobacillus plantarum*, C1, C2, and C3: Subsampling. Values are mean ± standard deviations, values with the different superscript alphabet in the same column show significantly different results ( $P < 0.05$ ).



FIGURE 1. Test results on selective media, 1: negative HEA medium; 2: Negative BGA medium; 3: positive BGA medium; 4: negative MacConkey medium and 5: positive MacConkey medium (n=3).

Table 5. Interpretation of biochemical test results (n=3).

| Sample                                | Medium          |          |                 |
|---------------------------------------|-----------------|----------|-----------------|
|                                       | TSIA            | LIA      | Urea Agar       |
| <b>A (Control)</b>                    |                 |          |                 |
| Egg shell (EA)                        | negative        | negative | Negative        |
| Egg white (WA)                        | negative        | negative | <b>Positive</b> |
| <b>B (<i>B. salmalaya</i> 139SI).</b> |                 |          |                 |
| Egg shell (EB)                        | negative        | negative | Negative        |
| Egg yolk (YB)                         | <b>positive</b> | negative | Negative        |
| <b>C (<i>L. plantarum</i>).</b>       |                 |          |                 |
| Egg shell (EB)                        | negative        | negative | Negative        |
| Egg yolk (YB)                         | negative        | negative | Negative        |

especially for *Shigella* and *Salmonella*. On HEA media, positively suspected colony *Salmonella* spp. showed blue-green colonies with or without black spots in the center after incubation at  $35 \pm 2^\circ\text{C}$  for 24 h. Using selected MacConkey media, it was found that egg white from control treatment and on eggshell and egg yolk from *B. salmalaya* 139SI probiotic treatment showed positive result for *Salmonella* spp., whereas the part of the eggs from control and *L. plantarum* probiotic treatment showed negative test results. Whereas for MacConkey media, a positive colony suspected of *Salmonella* spp. showed a colorless colony after incubation at  $35 \pm 2^\circ\text{C}$  for 24 h (**Figure 1**).

Table 5 shows the Interpretation of biochemical test results conducted. Biochemical tests were performed by streaking of isolates in TSIA, LIA, and Urea Agar. Based on test results using TSIA media, it was found that the samples were positive for *Salmonella* spp. detected in the egg yolks from *B. salmalaya* 139SI probiotic treatment, whereas the part of the eggs another showed negative test results. Biochemical testing using LIA media showed that various parts of egg examined including eggshell, egg white and egg yolk from each treatment were negative for *Salmonella* spp. in *B. salmalaya* 139SI and *L. plantarum* treatments. The Urea agar test on samples from control egg white showed a change in pink color (positive reaction), whereas the eggshells and egg yolks from *B. salmalaya* 139SI- treated and egg yolks from the control birds showed no change in color (negative reaction). On HEA media, positively suspected colony *Salmonella* spp. showed blue-green colonies with or without black spots in the center after incubation at  $35 \pm 2^\circ\text{C}$  for 24 h. Brilliant green dye contained in the media acts to inhibit the growth of Gram-positive bacteria and most of the Gram-negative bacteria are bacilli-shaped. BGA contains lactose and sucrose. Bacteria that have the ability to ferment lactose and sucrose will produce acids that change the color of the media from red to yellow. Bacteria that have the ability to ferment lactose and sucrose will form yellow or green colonies surrounded by yellow or green zones.

According to (Kodaka *et al.*, 2000) that *Salmonella* spp. does not have the ability to ferment lactose and sucrose. This causes *Salmonella* spp. to form a colony of red, pink, or nearly white and surrounded by pink media after incubation at  $35 \pm 2^\circ\text{C}$  for 24 h, as shown in Figure 1.

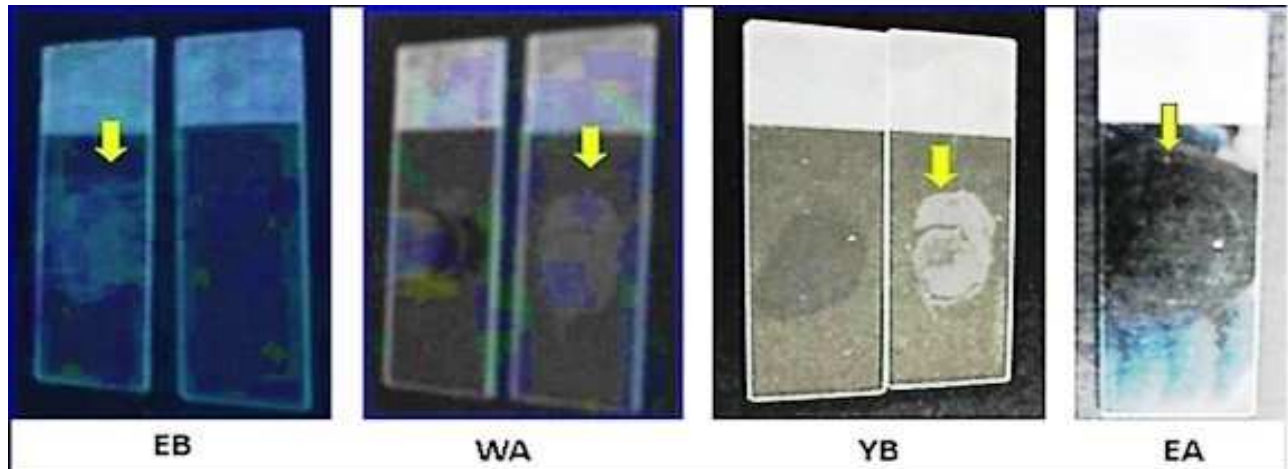
TSIA media is a medium for *Salmonella* biochemistry tests (Sakano *et al.*, 2013). This medium contains three types of carbohydrates, namely glucose, lactose, and sucrose. The bacteria that can ferment lactose and sucrose will change the color of the media from red to yellow in the slant part, whereas bacteria that can ferment glucose will change the color of the media to yellow in the butt part. The TSIA media also contains  $\text{FeSO}_4$  which acts as an  $\text{H}_2\text{S}$  gas detector produced by the bacteria tested. Bacteria that can produce  $\text{H}_2\text{S}$  will form black deposits. *Salmonella* can ferment glucose but cannot ferment lactose and sucrose. This is what causes *Salmonella* to form red in the slant and yellow in the bottom. *Salmonella* has the ability to produce  $\text{H}_2\text{S}$  gas (Yi *et al.*, 2014) so that it forms black in the stab area. The media contains lysine which acts as a substrate to detect the presence of the enzymes of lysine decarboxylase and lysine deaminase. *Salmonella* also can produce the enzyme lysine decarboxylase, which causes the bottom of the slants to turn purple.

Figure 3 describes the outcomes of the serological test conducted. *Salmonella enterica* serovar *Enteritidis* contamination is frequently investigated since it is the predominant serotype implicated in foodborne infections (salmonellosis) caused by egg or egg product ingestion. Serotyping was tested on eggs samples suspected of being infected by *Salmonella* from previous tests. Based on the results of the serotyping test, it was found that the overall samples examined did not form a lump (negative test reactions), indicating the absence of *Salmonella* spp. in that egg section. The *S. enteritidis* serotyping test was used to classify *Salmonella enterica* subsp. subtype enterica (*Salmonella* Subcommittee of the Nomenclature Committee of the International Society for Microbiology, 1934). The Kauffmann-White-Le Minor (KW) technique was used for *Salmonella* serotyping,



which is a bacterial agglutination scheme using a particular serum. Serotyping characterizes an organism's antigenic organization by recognizing somatic antigens (O) and flagellar antigens (H) by responses with specific antiserum. These antigens are highly variable, consisting of 64 of antigen somatic (O) and 114 of the flagellar

variants (H) that have been identified (Grimont and Weill, 2007). Autoagglutination and loss of antigen expression as observed in coarse strain, non-motile, and mucoid, can sometimes cause unbearable tension, but these strains usually have little epidemiological significance.



**FIGURE 3.** Serological test results for control group: egg shell (EA) and egg white (WA) and *B. salmalaya* 139SI group: egg yolk (YB) and eggshell (EB). (n=20 per group). The serological results were negative for all groups.

**The detection method using 16S rRNA:** The results of *Salmonella*-free egg testing by the PCR method are presented in **Figure 4**. The next step was to test *Salmonella*-free eggs (*S. enteritidis*) using the PCR method. Based on the results, it was found that various parts of the egg examined including eggshells, egg whites and egg yolks from each treatment showed negative test results, i.e there was no indication of *Salmonella* spp. contamination in probiotics treatment group. Based on *Salmonella*-free eggs test results, obtained differences in results between conventional tests with molecular identification testing using the PCR method. Based on the quantitative polymerase chain reaction (PCR) test results, it was found that overall, the parts of the eggs examined including eggshells, egg whites and egg yolks from each treatment showed negative test results, indicating the absence of *Salmonella* spp.

When the results of *Salmonella*-free egg tests were compared, disparities between traditional tests and molecular identification testing using the PCR technique occurred. These discrepancies urge an investigation of putative variables contributing to result variability. The quantitative polymerase chain reaction (PCR) findings indicated that the analyzed sections of the eggs, which included eggshells, egg whites, and egg yolks from each treatment, all tested negative. This indicates that *Salmonella* spp. are not present in the egg's components. The disparity in findings between traditional testing and PCR might be attributed to variations in the sensitivity

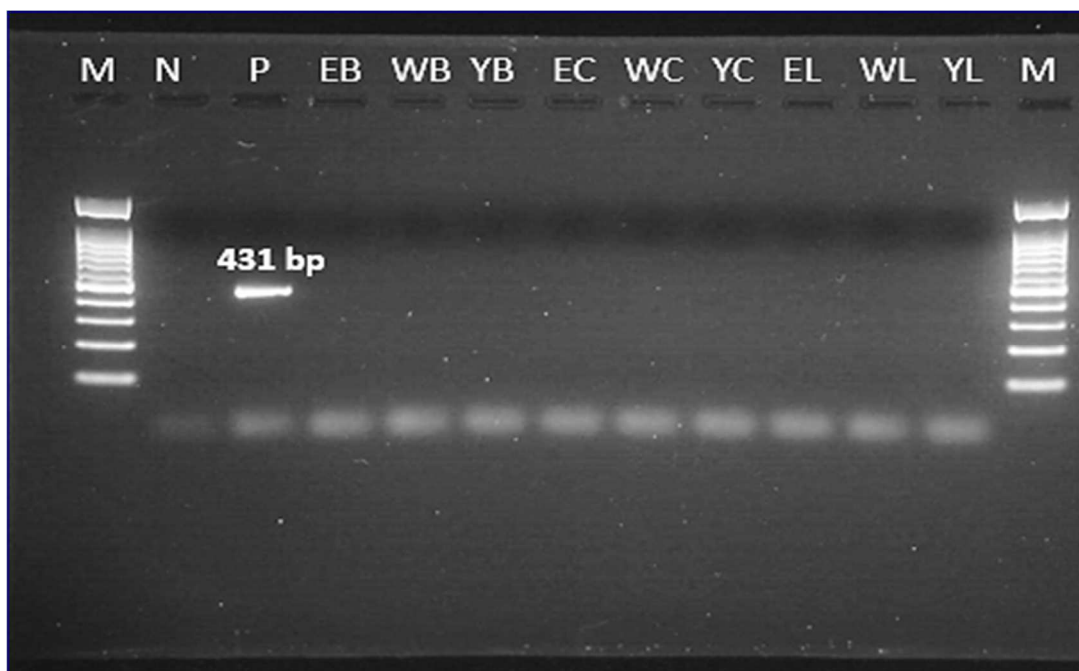
and specificity of the two procedures. As a very sensitive molecular technology, PCR may identify low quantities of genetic material even when bacteria are no longer alive. Conventional tests, on the other hand, focus on bacterial growth characteristics and metabolic activities, which might result in false-negative findings if the bacterial load is below the detection threshold or the bacteria are in a non-viable state (Ioos *et al.*, 2010; Merckx *et al.*, 2017).

Therefore, test results are relatively inaccurate and molecular identification confirmation is therefore required to obtain the accuracy of identification and higher levels of sensitivity. From our experimental results, it was shown that probiotics administration of both *L. plantarum* and also *B. salmalaya* 139SI (Ajeng *et al.*, 2021; Ismail *et al.*, 2022) can produce *Salmonella*-free eggs on laying hens. However, among the treatments tried, the probiotic treatment using *L. plantarum* was more promising. This is based on Probiotics of *L. plantarum* originated from *dadih* Kerinci have been proven to be effective in controlling the case of Salmonellosis (*S. enteritidis*) in the commercial poultry farming industry, especially in the laying hens farming industry. In the future, integrating multiomics platforms could be valuable for identifying illness progression or early disease diagnosis (Ajeng *et al.*, 2022; Baharum *et al.*, 2023; Radian *et al.*, 2022). Probiotics use a multifaceted strategy to reduce *Salmonella* development in layer hens. These beneficial bacteria compete with



*Salmonella* in the gastrointestinal system for resources and attachment sites, create antimicrobial compounds, increase the host immune response, and improve gut barrier function. Probiotics also help with competitive exclusion factors, alter the makeup of the gut microbiota, and minimize stress-induced alterations in the gut environment. Probiotics use these processes to generate an environment in the digestive tract of layer hens that is less favorable to *Salmonella* growth and colonization, offering a natural and effective approach of avoiding *Salmonella* infection in eggs (Doyle and Erickson, 2012;

Gast *et al.*, 2022; Padgett, 2021). From our experimental results, it was found that probiotic administration of both *L. plantarum* and *B. salmalaya* 139SI can produce *Salmonella*-free eggs on laying hens. The probiotics treatment of *L. plantarum* is the best treatment compared to the others. This is based on the test results of *Salmonella*-free eggs showing negative test results, i.e no *S. enteritidis* was found on each part of the examined eggs, namely eggshells, egg white and egg yolk, using both conventional test results and also molecular identification tests PCR method.



**Figure 4.** Visualization of DNA electrophoresis from PCR products to detect *S. enteritidis*. M: Marker; N: Negative control; P: Positive control *S. enteritidis* code number 4301/15; EB: Eggshell *B. salmalaya* 139SI; WB: Egg white *B. salmalaya* 139SI; YB: Egg yolk *Bacillus salmalaya* 139SI; EC: Eggshell Control; WC: Egg white Control; YC: Egg yolk Control; EL: Eggshell *Lactobacillus plantarum*; WL: Egg white *Lactobacillus plantarum* and YL; Egg yolk *Lactobacillus plantarum*.

**Conclusion:** This study evaluates the potentials of two bacteria from *L. plantarum* and *B. salmalaya* as probiotics against the infection of *Salmonella* in chicken layers. The supplementation of probiotics *L. plantarum* and *B. salmalaya* 139SI into drinking water resulted in *Salmonella*-free eggs in laying hens. This was evidently observed in the parameters observed include increased in chick layer body and eggs weight, and absence in all the biochemical and PCR tests conducted. Overall, *L. plantarum* was a suitable probiotic candidate to be administered or integrated into the feeds of newly hatched chicks to reduce *Salmonella* occurrence in the chicken layer based on the promising outcomes in several tests performed on the egg samples obtained from the laying hens in this study.

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