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Honors Thesis submitted to Shackouls Honors College, Mississippi State University, Spring 2022

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"The really good ideas…, you never do them by yourself, it is always with a room full of people with different ideas: arguing, collaborating, and sharing" – Dr. Genevieve Bell.

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INTRODUCTION

Necrotic enteritis (NE), caused by *Clostridium perfringens* (CP), is an emerging disease that costs approximately a six-billion-dollar loss annually to the poultry industry worldwide (Van Der Sluis, 2000; Wade and Keyburn, 2015). NE is a disease that causes various levels of necrosis in the small intestine and results in 2-50% mortality in chickens. The economic loss is not only related to an increase of daily mortality but also it affects the weight gain and feed conversion rate (Paiva and McElroy, 2014). For the treatment of NE, antibiotics are effective. However, low level of antibiotics added to animal feed as an antimicrobial growth promoter (AGP) has been shown to be an effective measure to prevent the disease (Paiva and McElroy, 2014). Due to the extensive belief that antibiotic-resistant bacteria found in humans are associated with antibiotics used in animals, many poultry companies have removed growth promoters from poultry feed, resulting in an increased incidence of necrotic enteritis. Consumers often misunderstand the difference between growth promoters and growth hormone, labeling antibiotic free or hormone free has become a marketing strategy to ease consumer's concern. Although the origin of

antibiotic-resistant strains from animals is debatable, a trend of limiting usage of antibiotics in poultry and livestock is definitive. The major concern by consumers, science community, and regulatory agencies is that antibiotic resistant bacteria will somehow transfer from livestock to humans. The residual antibiotics in meat products may affect the bacteria in humans to develop the resistance to antibiotic treatments. The antibiotic resistance becomes a problem in the healthcare system because the current medical field has limited antibiotic choices to treat antibiotic-resistant bacterial infections. Reducing antibiotic use in animals is the trend for livestock production; however, there is an urgent need to find alternative ways to care for the well-being of poultry and animals. Clearly, an alternative treatment without using antibiotics to treat diseases is critical for the survival and profitability of the poultry industry. The poultry industry must adapt to the new trend of reducing antibiotic usage to secure public opinion as well as to ease the concern of antibiotic resistance that is associated with the consumption of meat products.

Predisposing factors and the presence of mucin enhance the incidence of necrotic enteritis. Induction of necrotic enteritis in chickens is complex and difficult and it cannot be completed without the contribution of predisposing factors such as the presence of coccidia and dietary issues that damage the mucus of the intestinal wall (Prescott et al., 2016; Timbermont et al., 2011; Van Waeyenberghe et al, 2016). Non-starch polysaccharides and undigested nutrients can slow the intestinal movement and then stimulate intestinal epithelial cells to increase the production of mucin that supports the growth of anaerobic *C. perfringens* (Kleessen et al., 2003; Shojadoost et al., 2012).

Established by numerous studies, a healthy gut microbiome is essential to maintaining the health of animals and humans alike (Gaucher et al., 2015). *Lactobacillus* species have been

shown to improve the gut integrity of humans and animals to compete against pathogens (Ouwehand et al., 2016). Moreover, the presence of *Lactobacillus* has been shown to reduce the numbers of *Clostridium* in the human gut (Tomova et al., 2019). *C. perfringens*, the causative organism of necrotic enteritis, is an anaerobic bacterium that is often found in the gastrointestinal tract of avian species in a relatively small quantity. At low population levels, *Clostridium* is non-pathogenic but an increase in *C. perfringens* in the chicken gut can cause necrotic enteritis (Cooper and Songer, 2009; Stanley, et al., 2014). In addition, organic acids have been used to treat and prevent harmful bacteria such as *Campylobacter* spp. in chickens (Chaveerach et al., 2004). The hypothesis of this study was that the use of *Lactobacillus* species can inhibit the growth of harmful bacteria in the chicken gut and promote gut health. The objective of this study was to evaluate the effectiveness of probiotics bacteria, *Lactobacillus johnsonii* and *Lactobacillus salivarius*, to protect chickens against *Clostridium perfringens*.

Materials and Methods

Strain and cultivation.

A netB toxin positive type A *C. perfringens* strain was kindly provided from Dr. John F. Prescott. *Lactobacillus johnsonii* (LJ) and *Lactobacillus salivarius* (LS) strains were purchased from the American Type Culture Collection (ATCC). Before inoculation of the birds, the bacteria were cultured in a thioglycolate broth while *Lactobacillus* species was cultured in Brain Heart Infusion broth (Sigma-Aldrich). *Lactobacillus* species and *Clostridium perfringens* were cultured at 37°C for 18h under anaerobic environment. The cultures were placed in an BD Difco™ GasPak™ EZ and then incubated at 37°C.

In-vitro experiments

Lactobacillus johnsonii was cultured in brain heart infusion broth for 18 h and then the supernatant was collected from day 1 to day 6 and used for further testing. The supernatant from LJ culture was tested for acidity and the inhibition ability against CP (Figure 1). Acetic acid was diluted 2x, 10x, and 100x, and then was also used to test the inhibition ability against CP in presence or absence of 0.5% mucin (Figure 2). The mucin facilitates the growth of CP in the intestine. the concentration of acetic acid was 0.67, 0.335, 0.067 and 0.0067 mM.

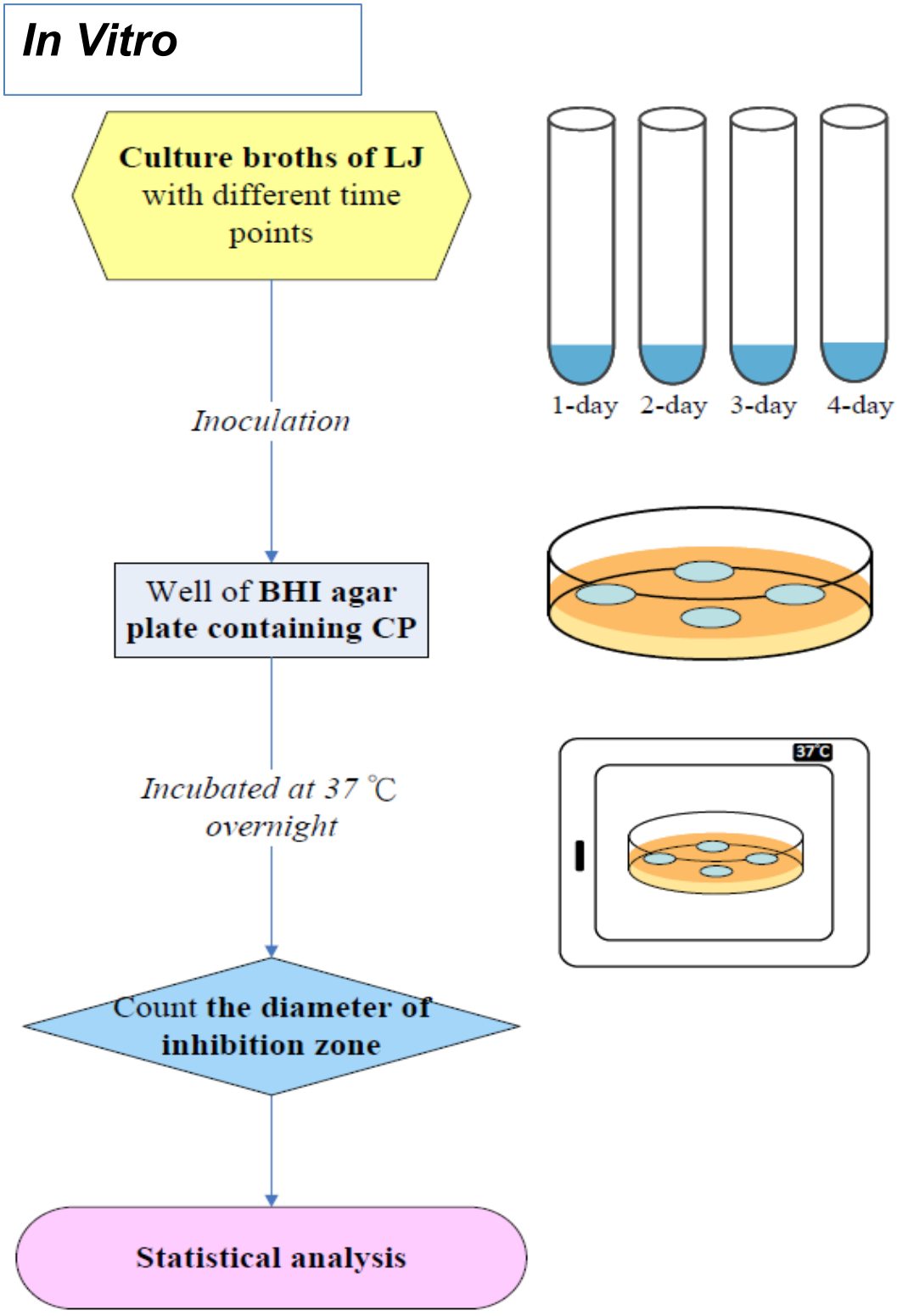


Figure 1. Testing acidity of *Lactobacillus* and inhibition of the growth of *Clostridium perfringens* by *Lactobacillus*.

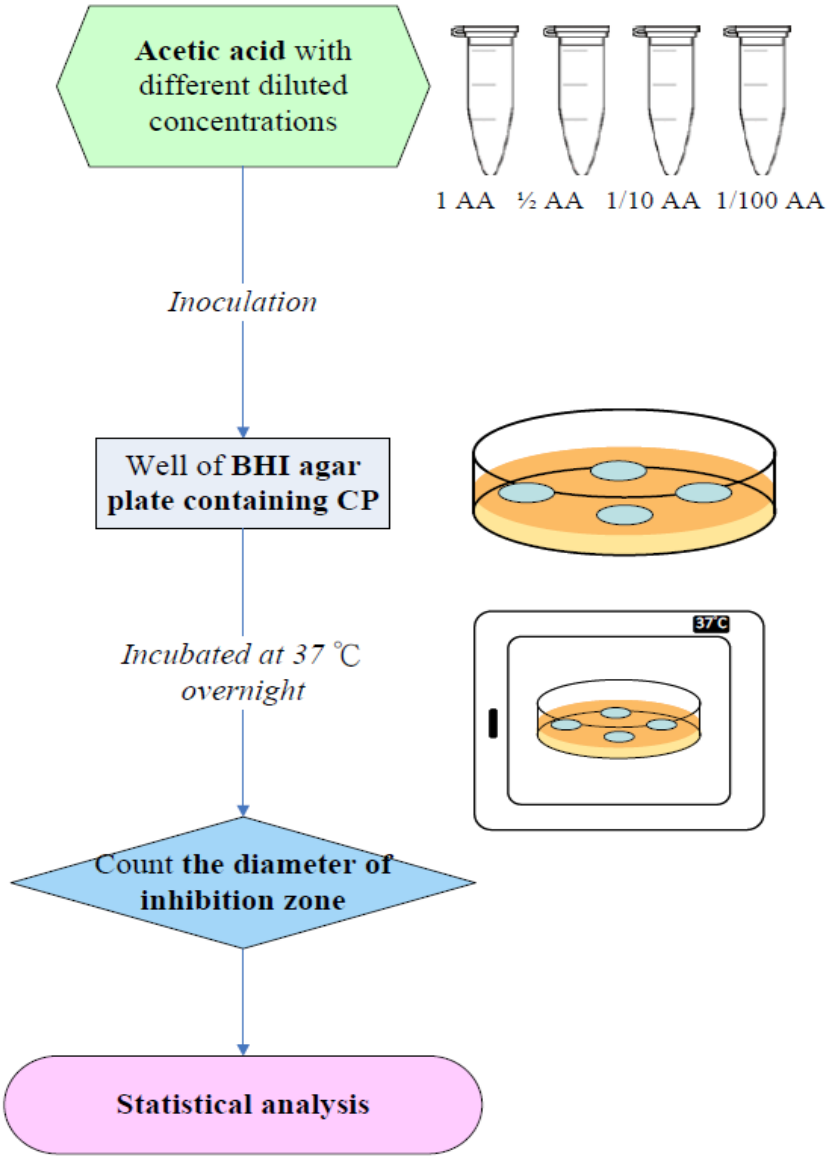


Figure 2. Testing the effects of acetic acid on the inhibition of *Clostridium perfringens*

Evaluation of *Lactobacillus* against *Clostridium perfringens* (CP) in chickens.

The specific aim here was to evaluate the effectiveness of probiotics bacteria, *L. johnsonni and L. salivarius*, to protect chickens against CP. In the experimental design, broiler chicks kindly provided by a commercial hatchery were divided into four groups: A) positive control (CP challenge, no treatment, B) *L. johnsonii* treatment +CP challenge, C) *L. salivarius* + CP challenge, and D) negative control (no treatment, no challenge). Groups A to C were challenged with CP (Table 1). The challenge model was conducted as described in Figure 3. LS and LJ (2 x 108 CFU/birds) were administered by drinking water and oral gavage for three days from day 8 to day 10, respectively. CP (2 x 10^8 CFU per bird) was mixed in feed and given three times a day from days 15 to 18. On day 19, all birds were euthanized, and the lesion of intestine was scored for NE. The lesion was scored as described by Yang et al. (2019). Number 1 means the normal gut; numbers 2 to 4 represent the severity of intestinal necrosis. Number 4 is the most severe level of lesions. If lesion score is greater than or equal to 2, the bird is considered as positive for NE.

Chicks were randomly grouped and placed in separate iron-made tanks with nets on the top in a BSL-2 animal facility. Feed and water were provided *ad libitum*. Natural Chick Starter/Grower feed containing 18% protein without antibiotics was used to feed chicks for the first 7 days. The feed was further mixed with fishmeal 60N (Seven Springs Farm, Check, Virginia, USA) at a ratio of 1:1 to form wheat-based diets as fishmeal diet.

Table 1. Groups of treatments and control groups

Group	Probiotics	Challenge	# of chickens	Samples
A	**No**	**Cp +** ***Eimeria***	26	Intestinal contents
B	***Lactobacillus salivarius***	**Cp +** ***Eimeria***	26	Intestinal contents
C	***Lactobacillus johnsonii***	**Cp +** ***Eimeria***	21	Intestinal contents
D	**No**	**No**	26	Intestinal contents

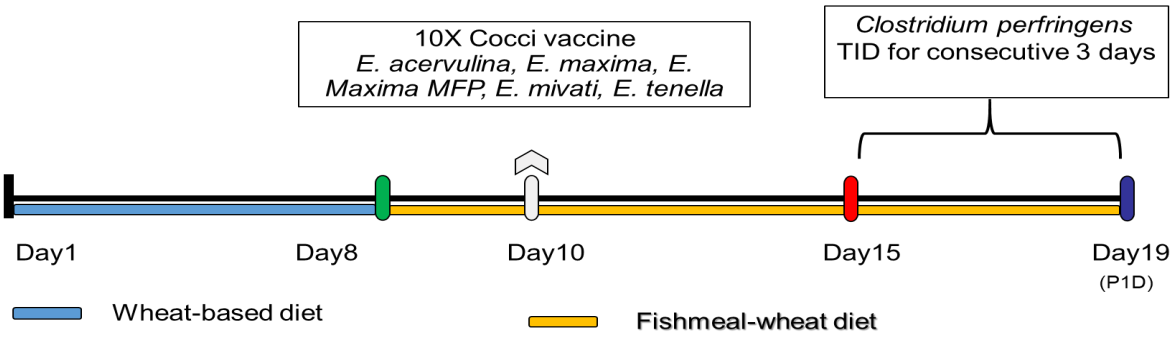


Figure 3. *Clostridium perfringens* challenge model. Chickens were fed wheat-based diet from day 1 to day 8 and changed to fishmeal-wheat diet from day 9 to day 19. Ten-fold dose of a coccidial vaccine containing *Eimeria acervvulina, E. maxima, E. maxima* MFP*, E. mivati, and E.*

tenella was given orally at day 10. *Clostridium perfringens* was mixed in wheat-based diet containing 2 x 10^8 CFU per bird three times per day from day 15 to 18. Necropsy and lesion scoring were performed at day 19.

RESULTS

Characteristics of *Clostridium perfringens* and *Lactobacillus johnsonii*

Clostridium perfringens grown anaerobically on a blood agar plate exhibited a double (α and β) hemolytic zone. Using gram staining, CP showed purple bacteria as gram-positive bacterium (Figure 4). *Clostridium perfringens* is at the root cause of Necrotic Enteritis in chickens, occurring primarily in the intestinal tract.

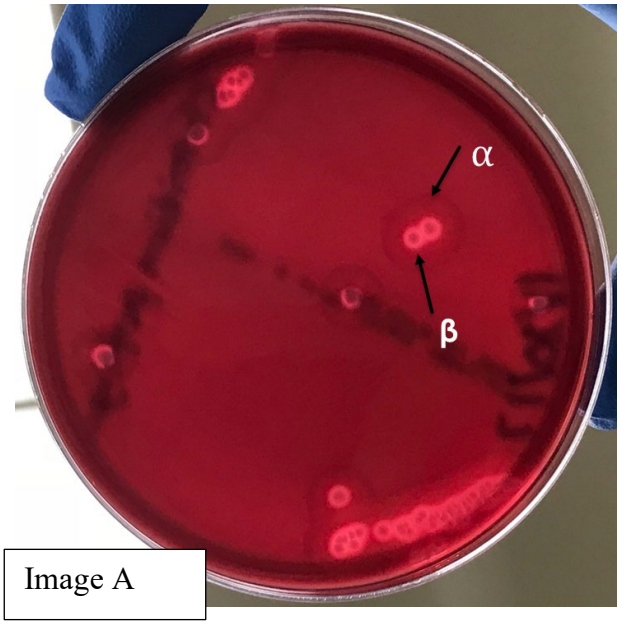


Image A

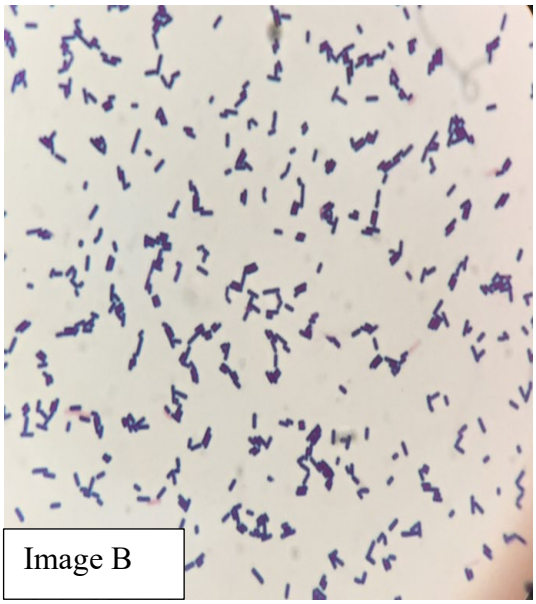


Image B

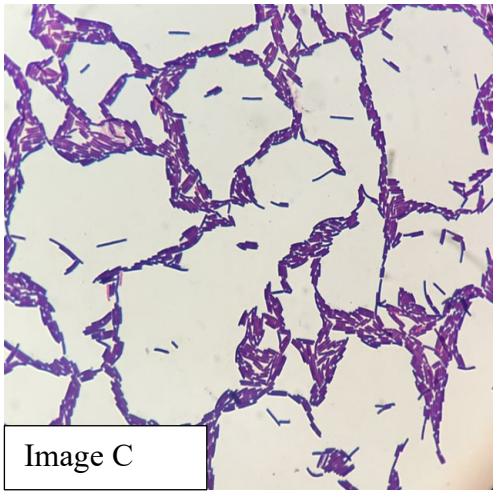
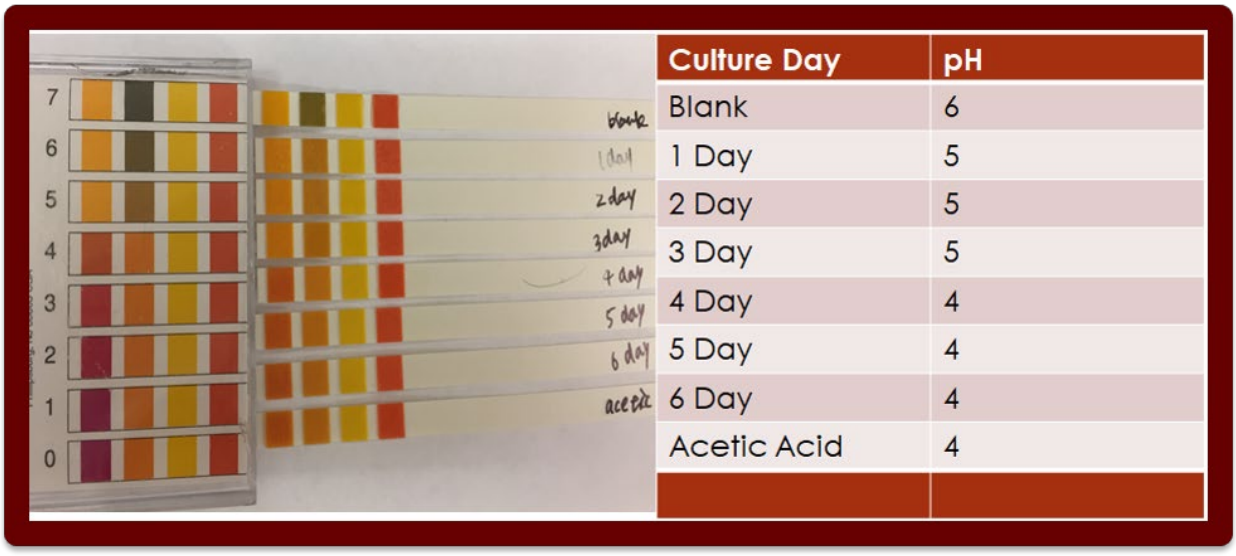


Image C

Figure 4. Images of bacteria. *Clostridium perfringens* grown on a blood agar plate (A); CP showed as gram-positive rods (B); Gram-positive *Lactobacillus* rods (C).

***In-vitro* study**

The supernatants were collected from *Lactobacillus johnsonii* cultures, and the pH was measured from Day 1 to day 6. This was an important notion to analyze, as the pH levels relate towards the formation of colonies in the gut integrity for *Lactobacillus johnsonii.* The pH decreased as the incubation time increased. The pH reached to 4 after 4-day incubation and remained at 4, and the pH of acetic acid was 4 (Figure 5).



Culture Day	pH
Blank	6
1 Day	5
2 Day	5
3 Day	5
4 Day	4
5 Day	4
6 Day	4
Acetic Acid	4

Figure 5. The pH of supernatant from *Lactobacillus johnsonii* and acetic acid was determined using the pH paper which ranged from pH 0 to 7.

The inhibition zone of the supernatant of LJ against CP increased as the incubation time increased in the presence or absence of mucin (Figure 6). However, the presence of mucin did decrease the inhibition zone when it compared to those without mucin. Mucin is a protein associated with the intestinal tract of chickens, and the presence of mucin facilitate the growth of CP in the intestine (Collier et al., 2008). The supernatant of LJ after 4 days of incubation maximizes the inhibition of CP growth in both mucin and non-mucin conditions.

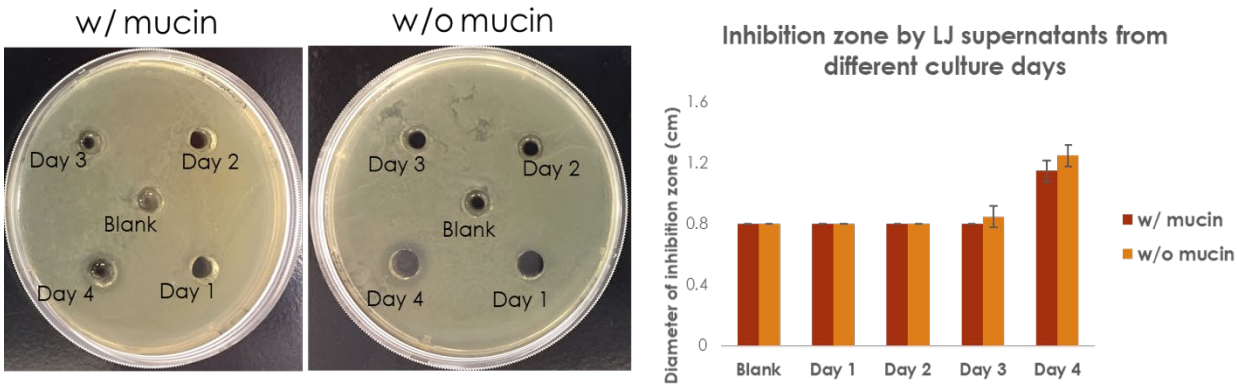


Figure 6. The inhibition zones of *Lactobacillus johnsonii* against *Clostridium perfringens* in presence or absence of mucin.

Acetic acid at the concentration of 0.67 mM, pH 4 showed the maximal inhibition compared to other diluted concentrations (2x, 10x, and 100x) in both mucin and non-mucin induced conditions (Figure 7). Two-fold dilution of acetic acid (0.335 mM) also show a clear inhibition zone but it is smaller than the zone inhibited by 0.67mM acetic acid.

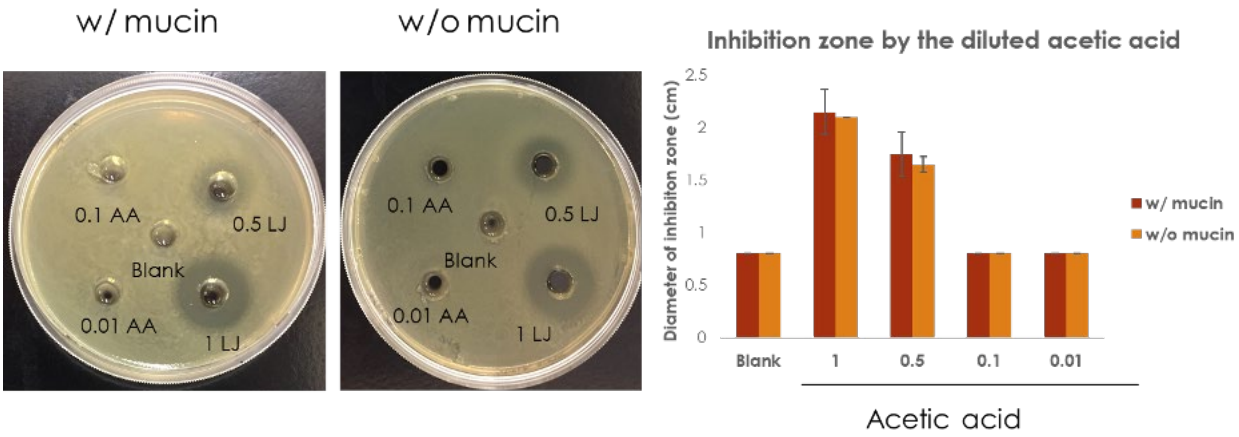


Figure 7. The inhibition zones of different concentrations of *acetic acid* against *Clostridium perfringens* in presence or absence of mucin.

In this *in-vivo* study, L. *johnsonni* or *L. salivarius* supplement did not protect birds against the CP challenge, the mortality in probiotic treatment groups was higher than the group without the treatment (Table 2). Orally giving probiotics daily seems to introduce stress to the birds and increase the susceptibility of CP, resulting in higher mortality and intestinal lesions than the positive control group (Challenged but without *Lactobacillus* treatment).

Table 2. Incidence and mortality of necrotic enteritis after CP challenge

Group	# of chickens	NE case	**NE Incidence**	# of death	**NE mortality**
No treatment CP + coccidia	26	5	**19.23%**	2	**7.69%**
Lactobacillus salivarius CP+ coccidia	26	13	**50.00%**	4	**15.38%**
Lactobacillus johnsonii CP+ coccidia	21	12	**57.14%**	9	**42.86%**
Negative control	26	0	**0.00%**	0	**0.00%**

Based on the lesion score of necrotic enteritis in different groups, the results were consistent with the results from Table 2. The lesion score of the two *Lactobacillus* treatment groups after CP challenge were worse than the challenge group without any *Lactobacillus* supplements (Refer to Figure 8).

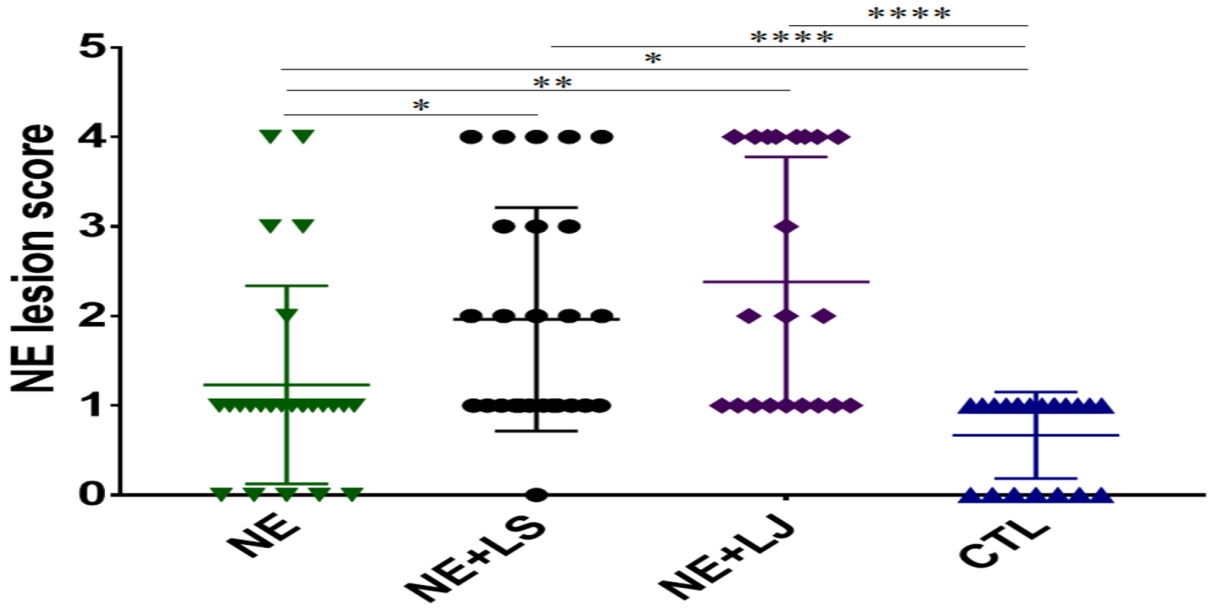


Figure 8. Dot density graph of lesion score for necrotic enteritis (NE) in chickens using Prism software. The score equal or greater than 2 is considered positive for NE. The data represents the mean NE lesion score $\pm$ SD. *$p < 0.05$, **$p < 0.01$

DISCUSSION

Probiotics and prebiotics have been considered as supplements to improve the gut microbiota and intestinal integrity. Regardless, numerous studies have been done; however, the effects of probiotics and prebiotic on chicken gut are still inconclusive. In this study, *in-vitro* experiments indicated that *L. johnsonii* and acetic acid have the potential to be used as a probiotic and additive, respectively, to inhibit the growth of CP. However, *in-vivo* experiments or animal challenge studies did not show any effectiveness of *L. johnsonii* or *L. salivarius* at these experimental conditions. Although the current study did not prove these probiotics are beneficial to chickens against *C. perfringens*, *L. johnsonni, L. salivarius* and *Bifidobacterium animalis* have been proven to be useful supplement to treat Type 1 diabetic patients (Wang, et al,2022). Moreover, the use of *L. salivarius* as a probiotic is beneficial to weight gain in chicken (Neveling and Dicks, 2021). The use of probiotics to improve the overall gut health has great potential to replace antibiotics used in chickens.

In vitro assay was not positively correlated with *in vivo* study. The treatment groups had more NE positive birds than the challenge group. These *Lactobacillus* strains used here did not prevent the disease and even increase the susceptibility to NE. Other reason may be due the daily administration of probiotics by oral gavage and the physical handling could have induced stress in the birds. Disturbing birds increases the susceptibility of birds to CP, resulting in higher mortality and severe intestinal lesions than the positive control group (challenged without probiotics). The other possibility is because the strains used here are not chicken-origin. Therefore, we consider isolating *Lactobacillus* species from the intestine contents from NE-resistant chickens in this trial. The other beneficial bacteria *Bifidobacterium* species can be another one to be used in our future studies.

The conclusion is that neither *L. johnsonii* nor *L. salivarius* protects chickens against the CP challenge. Different routes of administering the probiotics to avoid inducing stress to chickens, such as reducing harsh light and handling chicken calmly should be considered for future studies. Further studies by using mixtures of beneficial bacteria and organic acids that may have synergistic effects in the chicken intestine, should be investigated.

CONCLUSION AND FUTURE IMPLICATIONS

Multiple probiotic bacteria and organic acids should be tested for a synergistic effect, as an alternative prevention method to replace antibiotics. In addition, administering treatments should avoid any stress-inducing environments. Although the *in vitro* study using *L. johnsonii* and acetic acid showed inhibition zones around the well of agar plates, the application to an in vivo experiment translated into a rejection of the hypothesis. A combination of a mixed probiotic cultures and administration route of probiotics should be less invasive, using less stress induction during the process. Giving probiotics at day-old age or *in-ovo* injection also should be considered to allow beneficial bacteria to establish earlier in the gut to prevent the colonization of harmful bacteria.

RESEARCH COMPLIANCE

The Project IBC 003-17 was approved by Mississippi State University Institution of Biosafety Committee to use *Clostridium perfringens* for research. *Clostridium perfringens* CP54 was originally obtained from Dr. John Prescott, University of Guelph under the USDA/APHIS approved permit of VS-16-6. Chicken trials were approved by MSU IACUC.

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