THERAPEUTIC EFFECT OF COMMERCIAL PROBIOTIC LACTOBACILLUS ON AVIAN COLIBACILLOSIS

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Therapeutic Effect of Commercial Probiotic Lactobacillus on Avian Colibacillosis'** by Mohammad Alif Bin Bakri, and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 – Research Project.

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DEDICATIONS

I dedicate my thesis to my beloved lecturers and seniors who have supported me throughout the process. I appreciate Dr. Mohd Farhan Hanif Bin Reduan and Dr. Siti Nor Azizah Binti Mahamud, for their help in develop my skills as a veterinary student.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 5436 – Research Project

Avian colibacillosis caused by *Escherichia coli* is one of the most common diseases in the poultry industry, affecting poultry growth and development. The continued use of antimicrobial medications as treatment for avian colibacillosis has promoted the emergence of antimicrobial resistances toward *E. coli*. This study investigated the therapeutic effect of probiotic lactobacillus against *Escherichia coli* isolated from avian colibacillosis. Thus, probiotics were prepared and tested to check their viability and ability of the probiotic against the *E. coli*. The probiotic lactobacillus was successfully prepared in this study by providing good nutrients and a medium for the lactobacillus bacteria to grow and replicate. The prepared probiotics have low pH values, which indicates low pH values help the lactobacillus withstand an acidic environment like the stomach. The curd formation of probiotic milk test suggested that the lactobacillus were present and viable. The colonization test revealed the probiotic lactobacillus able to colonize the agar and occupied most of the spaces against the *E. coli*. Moreover, lower concentrations of the probiotic were able to produce a sufficient colonization zone and suppressed the *E. coli* colonization on the agar. In conclusion, the prepared probiotics in this study had a therapeutic effect against the *E. coli* and the potential for future commercialization to prevent and treat avian colibacillosis.

Keywords: *Avian Colibacillosis, Antimicrobial Resistance, Probiotic, Colonization Test*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Kolibasilosis ayam yang disebabkan oleh *Escherichia coli* adalah salah satu penyakit yang paling penting dalam industri ayam dengan menjejaskan pertumbuhan dan perkembangan ayam. Penggunaan berterusan ubat-ubatan antimikrobial sebagai rawatan untuk kolibasilosis ayam telah menggalakkan kemunculan rintangan antimikrob terhadap *Escherichia coli*. Kajian ini menyiasat kesan terapeutik probiotik lactobacillus terhadap *Escherichia coli* daripada kolibasilosis ayam. Oleh itu, probiotik telah disediakan dan diuji untuk memeriksa daya maju dan keupayaan probiotik terhadap *E. coli*. Probiotik lactobacillus telah berjaya disediakan dalam kajian ini dengan menyediakan nutrisi dan medium untuk bakteria lactobacillus berkembang dan membiak. Probiotik yang disediakan mempunyai nilai pH yang rendah, yang menunjukkan nilai pH yang rendah membantu lactobacillus menahan persekitaran berasid seperti perut. Ujian pembentukan dadih susu probiotik mencadangkan bahawa lactobacillus wujud dan berdaya maju. Ujian kolonisasi mendedahkan probiotik lactobacillus mampu mengkolonis agar dan membiak di kebanyakkan ruang agar terhadap *E. coli*. Selain itu, kepekatan probiotik yang lebih rendah mampu menghasilkan zon kolonisasi yang mencukupi dan menghalang kolonisasi *E. coli* di atas agar. Kesimpulannya, probiotik yang disediakan dalam kajian ini mempunyai kesan terapeutik terhadap *E. coli* dan potensi untuk dikomersialkan pada masa depan untuk mencegah dan merawat kolibasilosis ayam.

Kata kunci: *Kolibacilosis Ayam, Rintangan Antimikrob, Probiotik, Ujian Kolonisasi*

1.0 Introduction

Diarrhoea is one of the most prevalent diseases that impair the growth and development of poultry animal (Liang *et al*., 2021). It can be caused by *Escherichia coli* and cause considerable economic losses due to high mortality, high morbidity, low feed conversion rate and difficult in management. As everyone is aware, antibiotics are frequently utilised to boost development and reduce bacterial diarrhoea losses in the poultry industry (Liang *et al*., 2021). Antibiotic abuse or long-term usage, on the other hand, causes the emergence of drug-resistant strains, weakens the immune system, secondary infections, and drug residues, all of which have a number of negative effects on food safety and health for people as well as the environment (Dibner and Richards, 2005; M'Sadeq *et al.*, 2015; Lekshmi *et al.*, 2017). In order to prevent and cure bacterial diarrhoea in poultry, this condition unavoidably increases the demand for effective alternatives with high efficacy, minimal side effects, little residue, and resistance.

Avian colibacillosis predominantly affects broiler chickens between the ages of 4 and 6 weeks and is a primary source of high morbidity and mortality in the poultry industry, resulting in significant economic losses (Guabiraba and Schouler, 2015). Septicemia is a fatal symptom of this disease, characterises it in its acute form, whereas pericarditis, airsacculitis, and perihepatitis define it in its subacute form (Allan *et al*., 1993). These infections are typically acquired due to a secondary infection caused by mycoplasma or a virus (Droual *et al*., 1992). Many *E. coli* represents serogroups 01, 02, and 078 isolates usually associated with colibacillosis in poultry (Allan *et al*., 1993).

Probiotics are live bacteria that have beneficial impacts on the consumer's health (Reid *et al.*, 2003). Probiotics can support body immune regulation, resistance toward pathogen colonisation, increase integrity of gut, and growth. (Clavijo and Flórez, 2018). Lactobacillus is the most utilized sources of probiotics in poultry industry, and they serve a vital role in preserving their health (Patterson and Burkholder, 2003; Hossain *et al.*, 2015; Rathnapraba *et al.*, 2018). The probiotic bacteria can withstand the acidity of stamach environment and the bile acids, colonise the digestive tract, and outcompete other microorganisms inside the host (Murry *et al*., 2004). The species that are currently used in probiotic solutions are diverse and numerous. *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus plantarum, Streptococcus thermophilus, Enterococcus faecalis, Bifidobacterium spp.,* and *E. coli* are the most common bacteria.

2.0 Research problem

Antimicrobial medications are often used in the poultry industry to boost growth and to manage bacterial diarrhoea. However, prolonged use causes the emergence of drug-resistant strains, weakens the immune system, secondary infections, and drug residues, all of which have a number of negative effects on food safety and health for people as well as the environment (Dibner and Richards, 2005; M'Sadeq *et al.*, 2015; Lekshmi *et al.*, 2017). Thus, an alternative way to prevent these mentioned issues is in high demand.

To our knowledge, there are limited evidence on the therapeutic effects of commercial probiotic lactobacillus on avian colibacillosis. As a response, the aims for this research

is to determine the probiotic lactobacillus potential as a therapeutic agent in treating avian colibacillosis.

3.0 Research questions

3.1 Does the probiotic lactobacillus possess therapeutic effects against *Escherichia coli* isolated from avian collibacillosis?

4.0 Research hypothesis

4.1 Probiotic lactobacillus possesses a therapeutic effect against *Escherichia coli* isolated from avian collibacillosis.

5.0 Objectives

5.1 To identify the therapeutic effects of commercial probiotic lactobacillus against *E. coli* isolated from avian collibacillosis.

6.0 Literature review

6.1 Avian colibacillosis

6.1.1 Introduction of avian colibacillosis

Escherichia coli is widely known for its ability to cause a variety of diseases (Kazemnia *et al*., 2014). Infection with *E. coli* can present in various forms in turkeys and chickens, the most frequent of these is colibacillosis. One of the most often reported diseases in the poultry industry is avian colibacillosis, which is caused by avian pathogenic Escherichia coli (APEC) (Dziva & Stevens, 2008). Due to the considerable mortality and poor egg quality production in broiler and laying hen flocks, this disease give economically

important to poultry breeders. *Escherichia coli* infections significantly impact output and birdlife, particularly on remote farms where biosecurity and cleanliness are commonly ignored (Kazemnia *et al*., 2014).

Broiler chicks between the ages of 4 and 6 weeks are most commonly affected by avian colibacillosis and is a primary source of high morbidity and mortality in the poultry industry, resulting in significant economic losses (Guabiraba and Schouler, 2015). Septicemia is a fatal symptom of this disease, characterises it in its acute form, whereas pericarditis, airsacculitis, and perihepatitis define it in its subacute form (Allan *et al*., 1993). These infections are typically acquired due to a secondary infection caused by mycoplasma or a virus (Droual *et al*., 1992). *Escherichia coli* also can causes a variety of diseases outside of the digestive tracts such as meningitis, sepsis, urinary tract infections, osteomyelitis, cellulitis, wound infections, and colibacillosis (Xia *et al*., 2011; Obeng *et al*., 2012). A large number of *E. coli* represents serogroups 01, 02, and 078 isolates usually associated with colibacillosis in poultry (Allan *et al*., 1993). Several bacterial properties, including adhesiveness and iron acquisition mediated by the aerobactin system, have been associated with virulence (Allan *et al*., 1993).

6.1.2 Zoonotic properties of APEC

According to a recent study, APEC isolates with two or more virulence markers (Johnson *et al*., 2008). Several virulence genes discovered on the large virulence-plasmid ColV (ompT, hlyF, iss, iroN, and iutA) were connected to APEC strains, according to de Oliveira *et al*. (2015). Johnson *et al*. (2008) claimed that APEC strains that carry virulence genes may respond as virulence

reservoirs and zoonotic pathogens, infecting humans by spreading to other animals.

6.1.3 Route of infection

The natural path of infection for APEC is still unclear, despite the fact that the respiratory routes seem to be crucial entrance. Reports state that 10 % to 15 % of coliform from avian alimentary tract are thought to have potentially harmful APEC serotypes (Dziva and Steven, 2008). Extraintestinal translocation only happens when there are stressors present, and both type of *E. coli* (avirulent and virulent) have been observed to colonise and live well in the gut (Dziva and Steven, 2008).

The consequences of both strains of pathogen (pathogenic and commensal) coexisting in the gut environment are unclear, but it's possible that this might be a major source of APEC strains, as has been observed for enteropathogenic *E. coli* and atypical enterohaemorrhagic *E. coli* (Hornitzsky *et al*., 2005). APEC's site in intestinal gives a favourable potential for environmental dissemination and spread through faeces.

APEC already identified for ability to survive in dry environments, and dust particles in chicken barns can contain up to 106 *E. coli* colony-forming units per gram (Dziva and Steven, 2008). Systemic APEC infections are thought to be caused by inhaling this contaminated dust. Eggs infection can happen during laying or oviduct development, resulting in the embryo and early chick mortality. Salpingoperitonitis was one of common form of avian colibacillosis

reported in breeder chickens in the United Kingdom (Jordan *et al*., 2005), however, what impacts how this illness presents is unknown.

6.1.4 Virulence factor of APEC

Outer membrane protease (ompT), hemolysin (HlyF), serum survival (iss), siderophore (iroN), and iron transport (iutA) are 5 important virulence genes that have been identified as APEC's markers (de Oliveira *et al*., 2015; Jrgensen *et al*., 2019). There was evidence that at least one out of five genes was present in APEC isolates from hens with a clinical diagnosis of colibacillosis, according to Johnson *et al*. (2008).

6.2 Antibiotic resistance

A major global health problem in both human and veterinary medicine is the formation, spread, aggregation, and persistence of strains of dangerous bacteria that are antibiotic-resistant. Numerous therapeutic and nontherapeutic uses of antibiotics in humans and companion animals such as therapeutic, prophylactic, and subtherapeutic uses in animals diet to promote growth, have significantly increased the genetic factors on pathogenic and microbes bacteria, favouring the spread, accumulation, and persistence of antibiotic-resistant bacteria (Alali *et al*., 2008).

Antimicrobial drugs, including β-lactamases, aminoglycosides, and fluoroquinolones, are still the most common treatment of colibacillosis outbreaks (Kim *et al*., 2007). However, the long used of antimicrobial medications in poultry has promoted the development and survival of antibiotic-resistant *E. coli* in Korea (Unno *et al*., 2011). Because of the possible

spread of resistance genes from poultry bacteria to human bacteria, resistance in poultry bacteria may increase the risk of public health (Cavicchio *et al*., 2012).

From the result of the antimicrobial susceptibility test conducted by Kim *et al*. (2020), Isolation of 79 APEC produce high resistance toward ampicillin (66 isolates, 83.5%), nalidixic acid (52 isolates, 65.8%), tetracycline (51 isolates, 64.6%), ciprofloxacin and cephalothin (37 isolates, 46.8% for each) in an antimicrobial susceptibility test. Moreover, it also show high resistance toward the third-generation of antibiotics (cephalosporins, cefotaxime and ceftazidime) and fourth-generation antibiotics (cefepime) was found in 18 (22.8%), 14 (17.7%), and 5 (6.3%) isolates, respectively.

6.3 Probiotic lactobacillus

6.3.1 Introduction

Clostridia perfringens, *E. coli* and *Salmonella spp*. may colonize the gastrointestinal tract of chickens. *E. coli*, *C. perfringens* and *Salmonella spp.* are common foodborne bacteria that found in processed poultry products, and they can cause serious disease and even cause mortality in humans (Murry *et al*., 2004). Enterotoxigenic *E. coli* causes avian colibacillosis, a dangerous infectious disease that affects various types of hens (Cao *et al*., 2013; He *et al*., 2014). Because of the high mortality and morbidity rates due to avian colibacillosis, it lead to considerable economic losses in the global poultry industry every year (Lau *et al*., 2010).

Antibiotics such as colistin sulphate and enrofloxacin have generally been used to prevent or control colibacillosis. Antibiotics' effectiveness has been reduced due to the emergence and rapid spread of antibiotic-resistant bacteria, which may pose serious health hazards to humans (Asai *et al*., 2011; Belanger *et al*., 2011). To reduce the prevalence of colibacillosis and preserve the health of animals, alternative antimicrobials derived from natural sources are required.

Probiotics are non-pathogenic microbial feed supplements that provide health advantages to the host and have been proposed as antibiotic alternatives in food animals (Li *et al*., 2008; Gareau *et al*., 2010). According to Murry *et al*. (2004), probiotics are live microorganisms (such as bacteria, fungus, and yeast) that have therapeutic effects when ingested by animals which is able to act as treatment and able to prevent of diseases occurence. The probiotic bacteria must able to withstand the acidity of stamach environment and the bile acids, colonise the digestive tract, and outcompete other microorganisms inside the host (Murry *et al*., 2004).

Probiotic species from *Lactobacillus, Bacillus, Streptococcus, Enterococcus, Bifidobacterium, Candida, Aspergillus,* and *Saccharomyces* have been shown to improve broiler performance (Zulkifli *et al*., 2000), inhibition of pathogen and intestinal microflora modulation (Higgins *et al*., 2007), intestinal histological changes (Kabir *et al*., 2005; Samanya and Yamauchi., 2002; Chichlowski *et al*., 2007), immunomodulation (Apata, 2008), parameter of haemato-biochemical (Ashayerizadeh *et al*., 2009), improving the broiler meat sensory characteristics (Kabir *et al*., 2005; Pelicano *et al*., 2003) and encouraging broiler meat quality (Kabir *et al*., 2005).

According to Murry *et al*. (2004), lactic acid bacteria (LAB) have been shown to inhibit the growth of various enteric bacteria *in vitro*, including *Salmonella typhimurium, Staphylococcus aureus, E. coli, Clostridium perfringens,* and *Clostridium difficile*. They have been used to treat various gastrointestinal disorders in both humans and animals. The antibacterial action of lactic acid bacteria in the intestine is due to their primary metabolites, lactic acid and short-chain fatty acids (SCFA). *Lactobacilli* such as *L. casei, L. lactis, L. acidophilus, L. salivarius, L. helveticus* and other type of LAB found as animal intestinal normal flora (Murry *et al*., 2004).

The ability of LAB to create inhibitory compounds by metabolized ambient substrates has been related to their ability to prevent the growth of enteric bacteria *in-vivo* and *in-vitro*. Organic acids and short-chained volatile fatty acids (VFA) have been identified as some of these inhibitory compounds. LAB break down produce large amount of lactic acid from breakdown of carbohydrates, which reduced the surrounding pH value and prevent other bacteria growth (Murry *et al.*, 2004).

6.3.2 Mechanisms of Action of Probiotic

Probiotics have significantly decreased the occurrence and duration of diseases due to enhancement of direct inhibitory and colonisation resistance against infections. Pathogenic bacteria have been demonstrated to be inhibited by probiotic strains *in-vitro* and *in-vivo* in various ways (Kabir, 2009). Probiotics have four different ways of working in poultry: maintaining healthy intestinal microflora via competitive inhibition and antagonism behaviour (Kizerwetter-Swida and Binek, 2009); changing metabolism by increasing digestive enzyme

activity while reducing ammonia production and bacterial enzyme activity (Yoon *et al*., 2004); enhancing intake of feed (Awad *et al*., 2009); and stimulating the immune system (Apata, 2008).

6.4 Application of probiotic in colibacillosis trial

The study by Zhang *et al.* (2016) indicated that the average daily gain (ADG) and body weight (BW) were both reduced by the E. coli K88 challenge, along with the activity of the digestive enzymes and the function of the intestinal barrier. But when broiler chickens were given an E. coli K88 challenge, nutritional supplementation with C. butyricum reversed these findings and enhanced function of intestinal barrier, the immune response, and activity of digestive enzyme. On the effects of growth performance, immunological response, function of intestinal barrier and activity of digestive enzyme in broiler chickens challenged with E. coli, there was no discernible difference between the colistin sulphate antibiotic treatment and the C. butyricum probiotic therapy (Zhang *et al*., 2016).

Another research conducted by Redweik *et al*. (2020) show that probiotics did not synergistically enhance serum antibody responses, as demonstrated by the combination of probiotics and the live Salmonella vaccination in broiler chicken. However, strain-specific synergistic protection against APEC was seen in whole blood and replicated by better in-vitro and in-vivo protection against c7122. Additionally, the group receiving this combination of treatments did not exhibit Salmonella shedding in faeces at day 7. The maximum shedding was seen in other groups, indicating that this combination can successfully

lower the risk of infection and various harmful bacterial colonisation (Redweik *et al*., 2020).

7.0 Materials and methods

7.1 Stock culture of *E. coli*

The stock culture of *E. coli* was obtained from the Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The *E. coli* was isolated from the clinical case of avian colibacillosis. The bacteria were subcultured and maintained according to standard bacteriology procedure.

7.2 Preparation of probiotic

The materials for probiotic preparation were obtained and purchased in the local markets.

Materials: Coconut water (350 ml), rice yeast (22 g), sugar (85 g), molasses (350 ml), probiotic lactobacillus (320 ml), drinking water (3 l), tomatoes (100 g), pineapples (150 g), chicken feeds (150 g) and rice water (250 ml).

Probiotic lactobacillus solutions were mixed with three tablespoons of sugar and left for an hour in the container. Later, rice yeast, molasses, coconut water and three litres of drinking water were added to a container. The container was closed tightly and fermented for 14 days. A total of 750 ml of the fermented mixture was poured into two different containers, A and B, after seven days. Each container was added with 250 ml of rice water and 150 g of commercial chicken pellet feeds. Then, 200 g and 250 g of mashed tomatoes and pineapples were mixed into containers A and B, respectively and were left to ferment for 14 days.

7.2 Probiotic quality test

The probiotics were examined according to several characteristics such as smell, colour and pH value to evaluate the quality of the probiotic.

7.3 Probiotic milk test

The viability of the lactobacillus bacteria in the probiotic was determined by the formation of curds in milk. A total of 10 ml of probiotic A or B and 5 ml of cold milk were mixed and poured into the container. Both mixtures were left at room temperature for 48 hours. The curd formation was observed for both types of probiotic.

7.4 Colonization test

The colonization test is adapted from the antimicrobial susceptibility test. A colony of *E. coli* was taken from the cultured agar using an inoculating loop and smeared evenly onto the surface of Mueller-Hinton agar plate. Next, several empty disks were soaked with probiotic lactobacillus for 3 hours and 24 hours in different probiotic concentrations. After that, the probiotic soaked discs were placed onto the agar. Then, the plates were incubated, and as the bacteria grew on the plate's surface, the probiotic lactobacillus diffused into the agar. The colonization zone was measured in centimetres and recorded.

8.0 Results

8.1 Probiotic preparation

Observation of the probiotic lactobacillus was done and the result is shown in Figure 1. There was the presence of gram-positive and rod-shaped bacteria,

which indicate the lactobacilli. A mixture of the ingredients in preparing the probiotics, such as sugar, chicken commercial feed and vitamins from the fruits, provided the nutrients and a conducive environment for lactobacilli to grow and replicate.

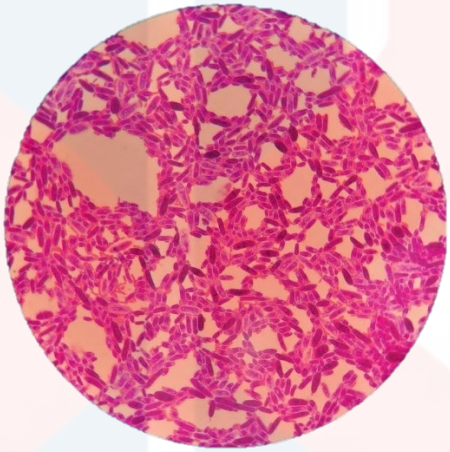


Figure 1 Probiotic lactobacillus under a microscope.
(Gram staining, 100x magnification)

8.2 Probiotic quality test

A probiotic quality test was performed based on the characteristics of colour, smell and pH value. Based on Table 1, both probiotics A and B showed light yellow colours resulting from the mixture of the ingredients during the probiotic preparation. Different smell findings were detected due to the different types of fruits used. Probiotic A produces a sour fermented smell from tomatoes, while probiotic B, which has pineapples, produces a sweet fermented smell. Both probiotics also produce low pH values (4.1 and 4.5), which are acidic.

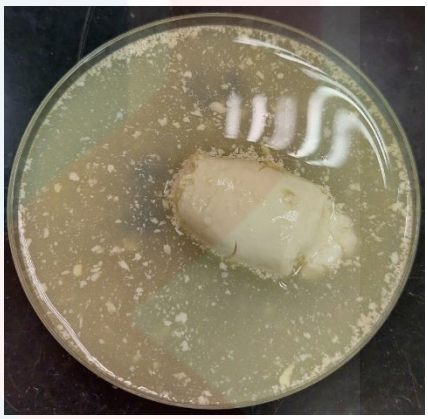
Table 1 Analysis of probiotic quality

Characteristic	**Probiotic A (Tomato)**	**Probiotic B (Pineapple)**
Colour	Light yellow	
Smell	Sour fermented smell	Sweet fermented smell
pH	4.1	4.5

8.3 Probiotic milk test

Besides, a probiotic milk test was performed, as shown in Table 2. After two days of resting in the room temperature environment, milk curd formation was observed in both probiotics. The formation of milk curd in a mixture of probiotic and fresh milk, demonstrated that the lactobacillus bacteria in probiotic were alive and viable.

Table 2 probiotic milk test

Probiotics	Results
A (Tomato)	The milk curdled after two days 
B (Pineapple)	

8.4 Probiotic colonization test

A probiotic colonization test was performed and the results are shown in Table 3 and Table 4. Both probiotics were able to produce colonization zones on the MHA plates against *E. coli*. From Table 3, 100% concentration of both probiotics showed larger colonization zones than other concentrations. 100% concentration of both probiotics produces colonization zones with a diameter of 2.5 cm and keeps increasing to 4.5 cm on day 5. The lowest concentrations, which were 6.25% also able to produce large colonization zones on day 5 for both probiotic A and B with a diameter of 3.0 cm and 2.5 cm, respectively. There were no huge differences in colonization zone size between the probiotic A and B for 3 hour soaking period.

For Table 4, 100% concentration of both probiotics demonstrates larger colonization zones compared to other concentrations. A hundred percent concentration of probiotic B produced the larger colonization zone, which was 5.0 cm, compared to the probiotic A on day 5 (4.0 cm) with a diameter of 2.5 cm (day 1) and keep increasing to 4.5 cm

on day 5. The lowest concentration, which was 6.25% also able to produce larger colonization zones on day 5 for both probiotics A and B with a diameter of 2.8 cm and 3.0 cm, respectively. There were no huge differences in the size of colonization zones between the probiotic A and B for 24 hours of soaking.

Table 3 Colonization zones of the probiotics on the MHA plates against the *E. coli* with a soaking period of 3 hours

Days	Concentrations (%)	Diameter (cm)	
		Probiotic A (mixed with tomato)	Probiotic B (mixed with pineapple)
1	100	2.5	2.5
	50.0	2.3	2.0
	25.0	2.0	2.0
	12.5	1.5	1.8
	6.25	1.5	1.5
3	100	3.5	3.5
	50.0	3.0	2.7
	25.0	2.7	2.5
	12.5	2.5	2.3
	6.25	2.5	2.1
5	100	4.5	4.5
	50.0	3.5	3.5
	25.0	3.5	3.1
	12.5	3.0	2.9
	6.25	3.0	2.5

Table 4 Colonization zones of the probiotics on the MHA plates against the *E. coli* of a soaking period of 24 hours

Days	Concentrations (%)	Diameter (cm)	
		Probiotic A (mixed with tomato)	Probiotic B (mixed with pineapple)
1	100	2.0	2.5
	50.0	1.8	2.5
	25.0	1.8	2.0
	12.5	1.8	1.8
	6.25	1.6	1.8
3	100	3.1	3.5
	50.0	2.5	3.3
	25.0	2.4	3.0
	12.5	2.3	2.7
	6.25	2.0	2.5
5	100	4.0	5.0
	50.0	3.5	4.8
	25.0	3.5	4.0
	12.5	3.3	3.5
	6.25	2.8	3.0

9.0 Discussion

9.1 Probiotic lactobacillus preparation

Lactobacilli are a group of gram-positive bacteria, rod-shaped, negative in catalase test and not forming spore bacteria of the family Lactobacillaceae. Lactobacilli bacteria produce lactic acid as the primary outcome ftom fermentation process. They need to be provided with carbohydrates, fatty acids or fatty acid esters, salts, nucleic acid derivatives, and vitamins due to their complicated dietary needs (De Angelis & Gobbetti, 2016). Therefore, lactobacillus needs to be provided with a medium with high nutrients and vitamins to grow and replicate. Most of the probiotic ingredients with high nutrients and vitamins, such as sugar, chicken feed, tomatoes and pineapples, were mixed for this study. Carbohydrates act as an energy source and assist with the fermentation process. Simple carbohydrates such as table sugar are made up of one or two sugars (monosaccharides or disaccharides) combined in a simple chemical structure that is easily utilized for energy (Holesh *et al*., 2021). Additional tomatoes and pineapples in the recipes also provide nutrients and vitamins for the lactobacillus. Tomatoes are rich sources of folate, vitamin C, potassium and vitamin E (Beecher, 1998). The main nutrients in pineapples are carbohydrates and water, which are also important sources of dietary fibre, minerals (manganese, magnesium, and copper), vitamins, organic acids (niacin, ascorbic acid, and thiamin), and sugars (Ancos *et al*., 2016),. A mixture of these ingredients provides a good nutrient medium for the probiotic lactobacillus to reproduce and be viable.

9.2 Probiotic quality test

The results demonstrated that the probiotics that have been produced have good qualities based on characteristics of colour, smell and pH value.s Both probiotics produce low pH values (4.1 – 4.5), which were acidic. This indicates that prepared probiotics can withstand the acidic environment like in the stomach. To survive passage through the stomach and small intestine, probiotic strains must tolerate the acidic and protease-rich conditions of the stomach and survive and grow in the presence of bile acids (Tuomola *et al*., 2001). The probiotic bacteria can withstand the acidity of stamach environment and the bile acids, colonise the digestive tract, and outcompete other microorganisms inside the host (Murry *et al*., 2004). Thus, the low pH is an important characteristic of a good probiotic. The smell and colour characteristics of the probiotic cannot be used as indicators of good probiotics because it depends on the methods and ingredients during the preparation process. For this study, tomatoes and pineapples, as one probiotic ingredient, can produce sour and sweet fermented smells, which were pleasant.

9.3 Probiotic milk test

A probiotic milk test was performed to identify the formation of curd from the mixture of probiotics and milk. Due to its ability to convert milk sugar (lactose) into lactic acid, lactobacillus bacterium (also known as *Lactococcus lactis*) is categorised as a LAB microorganism. The bacteria *L. lactis* employs enzymes to convert lactose into ATP when it is given to milk. Lactic acid is the byproduct of ATP synthesis. Milk is curdled by the lactic acid, which separates into curds and whey, which are used to make cheese. It also can lowering the product's pH and protecting it against the development of undesirable bacteria and moulds (Nuryshev and Stoyanova, 2016). In this study, both probiotics can form curd after leaving for two days in a room-temperature

environment. This indicates that the lactobacillus bacteria in both probiotics were actively alive and viable.

9.4 Probiotic colonization test

The ability of lactic acid bacteria to create inhibitory compounds by metabolizing ambient substrates has been related to their ability to inhibit the growth of enteric bacteria *in-vitro* and *in-vivo* (Murry *et al*., 2004). Lactic acid bacteria break down carbohydrates to produce large amounts of lactic acid, which lowers the pH of their surroundings and inhibits the growth of other bacteria (Murry *et al*., 2004). The colonization zone of the probiotic indicates one of the therapeutic aspects produced by the probiotic lactobacillus against the bacterial colonization on the agar. These colonization zones depend on the concentration of the probiotic and the soaking period of the disk. Theoretically, the disk with a higher concentration of probiotics produces a larger colonization zone.

The probiotic colonization test results revealed that 100% concentration of probiotic has larger colonization zones than other concentrations. The high concentration of probiotics has many probiotic cells in the disk that grows during the incubation. The longer soaking time allowed the probiotic to diffuse into the disks and produce a larger size of colonization zones. The colonization zones increased steadily over the number of days, indicating that these probiotics have high growth and replication properties and the ability to overgrowth on other pathogenic bacterial spaces, producing competition between these two bacteria for spaces on the agars. Moreover, the lowest probiotic concentration, 6.25% for both probiotics and soaking periods, also showed sufficient colonization zones on the agar, indicating that the probiotics were potent.

Numerous studies showed growth inhibitory effects of probiotics against various infections. Ota *et al.* (2009) revealed that yoghurt intake induces intestinal colonisation of probiotic bacteria like lactobacillus and provided circumstances to prevent colonisation of *Enterohemorrhagic E. coli* (EHEC). They discovered that several lactobacilli culture supernatants may inhibit the growth of many pathogens, including *Salmonella, Shigella, Staphylococcus aureus* and *Listeria monocytogenes* (Kargar *et al*,. 2009). Hassanzadazar *et al*. (2014) showed *Salmonella enteritidis, Bacillus cereus, Escherichia coli,* and *Listeria monocytogenesis* are all susceptible to the growth-inhibiting actions of the probiotics *Enterococcus fascium* and *Lactobacillus casei*. Another study conducted by Murry et al. (2004) indicates that lactic acid bacteria can prevent the growth of many enteric bacteria *in vitro*, including *Clostridium difficile., Staphylococcus aureus, Clostridium perfringens, E. coli* and *Salmonella typhimurium.* However, Karimi *et al* (2018) state that the probiotics had no inhibitory effects on the EHEC strain. Therefore, the therapeutic effects of probiotic lactobacillus against *E.coli spp.* are various because they depend on the species of lactobacillus bacteria selected for probiotic preparation and the strain of *E. coli* tested.

10.0 Conclusion

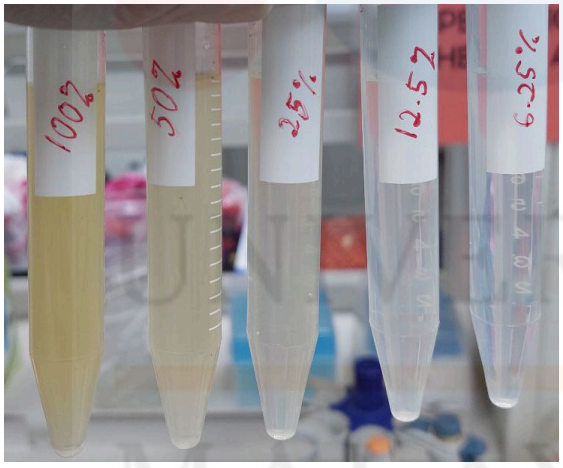
In conclusion, the present study prepared lactobacillus probiotics with good characteristics. Both probiotics prepared also can produce large colonization zones against *E.coli*. Thus, probiotic lactobacillus has therapeutic effects by limiting the growth spaces of *E.coli*.

11.0 Recommendations and future work

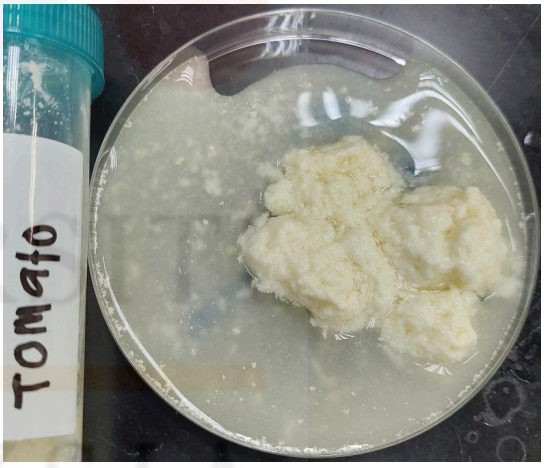
Several limitations were noted in this study. For the prospective study, the animal experiment on the therapeutic effect of probiotics lactobacillus needs to be done to determine the probiotic potency in the animal model. The results of the probiotics on the vital organs such as the liver, kidney and intestine need to be emphasized. Several procedures can be done to analyze the effects of probiotics in the live animal, such as haematological and serum chemistry analysis, gross and histopathological evaluation, and animal behaviour and abnormal signs. Next, the investigation of the adverse effects of probiotics on the animal can be done in future studies by increasing the probiotic dosage.

Appendices

Appendix A.1 Probiotic lactobacillus preparation



Appendix A.2 Probiotics titre dilution



Appendix A.3 Probiotic milk test

References

Alali WQ, Scott HM, Harvey RB, Norby B, Lawhorn DB, Pillai SD. Longitudinal study of antimicrobial resistance among Escherichia coli isolates from integrated multisite cohorts of humans and swine. Appl Environ Microbiol. 2008; 74(12):3672-81.

Allan, B. J., van den Hurk, J. V., & Potter, A. A. (1993). Characterization of Escherichia coli isolated from cases of avian colibacillosis. Canadian Journal of Veterinary Research = Revue Canadienne de Recherche Vétérinaire, 57(3), 146–151.

Ancos, B., S´anchez-Moreno, C., & Gonzalez-Aguilar, ´ G. A. (2016). Pineapple composition and nutrition. Handbook of Pineapple Technology: Postharvest Science, Processing and Nutrition, 221–239. https://doi.org/10.1002/9781118967355.ch12.

Apata, D.F. Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of lactobacillus bulgaricus. J. Sci. Food Agric. 2008, 88, 1253-1258.

Asai, T., K. Masani, C. Sato, M. Hiki, M. Usui, K. Baba, M. Ozawa, K. Harada, H. Aoki, and T. Sawada. 2011. Phylogenetic groups and cephalosporin resistance genes of Escherichia coli from dis- eased food-producing animals in Japan. Acta Vet. Scand. 53:52.

Ashayerizadeh, A.; Dabiri, N.; Ashayerizadeh, O.; Mirzadeh, K.H.; Roshanfekr, H.; Mamooee, M. Effect of dietary antibiotic, probiotic and prebiotic as growth

promoters, on growth performance, carcass characteristics and hematological indices of broiler chickens. Pakis. J. Biol. Sci. 2009, 12, 52-57.

Awad, W.A.; Ghareeb, K.; Abdel-Raheem, S.; Böhm, J. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 2009, 88, 49-56.

Belanger, L., A. Garenaux, J. Harel, M. Boulianne, E. Nadeau, and C. M. Dozois. 2011. Escherichia coli from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. FEMS Immunol. Med. Microbiol. 62:1–10.

Cao, G. T., X. F. Zeng, A. G. Chen, L. Zhou, L. Zhang, Y. P. Xiao, and C. M. Yang. 2013. Effects of a probiotic, Enterococcus faecium, on growth performance, intestinal morphology, immune response, and cecal microflora in broiler chickens challenged with Escherichia coli K88. Poult. Sci. 92:2949–2955.

Cavicchio, L., G. Dotto, M. Giacomelli, D. Giovanardi, G. Grilli, M. P. Franciosini, A. Trocino, and A. Piccirillo. 2012. Class 1 and class 2 integrons in avian pathogenic Escherichia coli from poultry in Italy. Poult. Sci 94:1202–1208.

Chichlowski, M.; Croom, W.J.; Edens, F.W.; McBride, B.W.; Qiu, R.; Chiang, C.C.; Daniel, L.R.; Havenstein, G.B.; Koci, M.D. Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, primalac, and salinomycin. Poult. Sci. 2007, 86, 1121-1132.

Clavijo, V., and M. J. V. Florez. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poult. Sci. 97:1006–1021.

de Oliveira, A. L., D. A. Rocha, F. Finkler, L. B. de Moraes, N. L. Barbieri, D. B. Pavanelo, C. Winkler, T. T. Grassotti, K. C. de Brito, B. G. de Brito, and F. Horn. 2015. Prevalence of ColV plasmid-linked genes and in vivo pathogenicity of avian strains of Escherichia coli. Foodborne Pathog. Dis. 12:679–685.12:679–685.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634–643.

Droual, R., Shivaprasad, H. L., Meteyer, C. U., Shapiro, D. P., & Walker, R. L. (1992). Severe mortality in broiler chickens associated with Mycoplasma synoviae and Pasteurella gallinarum. Avian Diseases, 36(3), 803–807. https://doi.org/10.2307/1591789

Dziva, F., & Stevens, M. P. (2008). Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. Avian Pathology, 37(4), 355–366. https://doi.org/10.1080/03079450802216652

Gareau, M. G., P. M. Sherman, and W. A. Walker. 2010. Probiotics and the gut microbiota in intestinal health and disease. Nat. Rev. Gastroenterol. Hepatol. 7:503–514.

Guabiraba, R., and C. Schouler. 2015. Avian colibacillosis: still many black holes. FEMS Microbiol. Lett. 362:1–8

Hassanzadazar H, Ehsani A, Mardani K. Antibacterial activity of Enterococcus faecium derived from Koopeh cheese against Listeria monocytogenes in probiotic ultra-filtrated cheese. Vet Res Forum. 2014;5(3):169–75.

He, C., B. Fu, P. Yi, X. Wei, Z. Yin, C. Lv, W. Zhang, X. Han, and X. Cun. 2014. Effectiveness of Xiang-Qi-Tang against Avian Pathogenic Escherichia coli. Pak. Vet. J. 34:127–129.

Higgins, J.P.; Higgins, S.E.; Vicente, J.L.; Wolfenden, A.D.; Tellez, G.; Hargis, B.M. Temporal effects of lactic acid bacteria probiotic culture on Salmonella in neonatal broilers. Poult. Sci. 2007, 86, 1662-1666.

Holesh, J. E., Aslam, S., & Martin, A. (2021). Physiology, Carbohydrates. In StatPearls. StatPearls Publishing.

Hornitzsky, M.A., Mercieca, K., Bettelheim, K.A. & Djordjevic, S.P. (2005). Bovine feces from animals with gastrointestinal infections are a source of serologically diverse atypical enteropathogenic Escherichia coli and Shiga toxin-producing E. coli strains that commonly possess intimin. Applied Environmental Microbiology, 71, 3405–3412.

Hossain, M. M., M. Begum, and I. H. Kim. 2015. Effect of Bacillus subtilis, Clostridium butyricum and Lactobacillus acidophilus endospores on growth performance, nutrient digestibility, meat quality, relative organ weight, microbial shedding and excreta noxious gas emission in broilers. Vet. Med-czech. 60:77–86.

Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan. 2008. Identification ofminimal predictors of avian pathogenic Escherichia coli virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46:3987–3996.

Jordan, F.T.W., Williams, N.J., Wattret, A. & Jones, T. (2005). Observations on salpingitis, peritonitis and salpingoperitonitis in a layer breeder flock. The Veterinary Record, 157, 573–577.

Kabir, S. M. L. (2009). The role of probiotics in the poultry industry. International Journal of Molecular Sciences, 10(8), 3531–3546. https://doi.org/10.3390/ijms10083531

Kabir, S.M.L.; Rahman, M.M.; Rahman, M.B.; Hosain, M.Z.; Akand, M.S.I.; Das, S.K. Viability of probiotics in balancing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. Biotechnology 2005, 4, 325-330.

Kargar M, Homayon M, Yaghoubi R, Manokianis A. Pathogenic gene stx1, stx2, eaeA and hly with Multiplex PCR in strains of E. coli O157: H7 isolated from children with acute gastroenteritis in the city of Shiraz. Iranian Journal of Infectious Diseases. 2009;14(44):7–12.

Kazemnia, A., Ahmadi, M., & Dilmaghani, M. (2014). Antibiotic resistance pattern of different Escherichia coli phylogenetic groups isolated from human urinary tract infection and avian colibacillosis. Iranian Biomedical Journal, 18(4), 219–224. https://doi.org/10.6091/ibj.1394.2014

Kim, T. E., Y. W. Jeong, S. H. Cho, S. J. Kim, and H. J. Kwon. 2007. Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic Escherichia coli isolates. J. Clin. Microbiol. 45:3309–3315.

Kizerwetter-Swida, M. and Binek, M. Protective effect of potentially probiotic Lactobacillus strain on infection with pathogenic bacteria in chickens. Pol. J. Vet. Sci. 2009, 12, 15-20.

Lau, G. L., C. C. Sieo, W. S. Tan, M. Hair-Bejo, A. Jalila, and Y. W. Ho. 2010. Efficacy of a bacteriophage isolated from chickens as a therapeutic agent for colibacillosis in broiler chickens. Poult. Sci. 89:2589–2596.

Lekshmi, M., P. Ammini, S. Kumar, and M. F. Varela. 2017. The food production environment and the development of antimicrobial resistance in human pathogens of animal origin. Microorganisms 5:11–25.

Li, L. L., Z. P. Hou, T. J. Li, G. Y. Wu, R. L. Huang, Z. R. Tang, C. B. Yang, J. Gong, H. Yu, X. F. Kong, E. Pan, Z. Ruan, W. Y. Xhu, Z. Y. Deng, M. Xie, J. Deng, F. G. Yin, and Y. L. Yin. 2008. Effects of dietary probiotic supplementation on ileal digestibility of nutrients and growth performance in 1- to 42-day-old broilers. J. Sci. Food Agr. 88:35–42.

Liang, W., Li, H., Zhou, H., Wang, M., Zhao, X., Sun, X., Li, C., & Zhang, X. (2021). Effects of Taraxacum and Astragalus extracts combined with probiotic Bacillus subtilis and Lactobacillus on Escherichia coli–infected broiler chickens. Poultry Science, 100(4), 101007. https://doi.org/10.1016/j.psj.2021.01.030

M'Sadeq, S. A., S. Wu, R. A. Swick, and M. Choct. 2015. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim. Nutr. 1:1–11.

Murry A. C., Jr., A. Hilton, Jr., and H. Morrison. 2004. Inhibition of Growth of Escherichia coli, Salmonella typhimurium, and Clostridia perfringens on Chicken Feed Media by Lactobacillus salivarius and Lactobacillus plantarum. International Journal of Poultry Science 3 (9): 603-607.

Nuryshev MZ and Stoyanova LG, (2016). New Probiotic Culture of Lactococcus lactis ssp. lactis: Effective Opportunities and Prospects. Journal of Microbial & Biochemical Technology, 2016. 8(4): 290-295.

Obeng AS, Rickard H, Ndi O, Sexton M, Barton M. Antibiotic resistance, phylogenetic grouping and virulence potential of Escherichia coli isolated from the faeces of intensively farmed and free range poultry. Vet Microbiol. 2012 Jan;154(3-4):305-15.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627– 631.

Pelicano, E.R.L.; de Souza, P.A.; de Souza, H.B.A.; Oba, A.; Norkus, E.A.; Kodawara, L.M.; de Lima, T.M.A. Effect of different probiotics on broiler carcass and meat quality. Br. J. Poult. Sci. 2003, 5, 207-214.

Rathnapraba, S., P. Kanagaraju, and K. Vijayarani. 2018. Effect of dietary supplementation of probiotic and BMD on the growth performance of broiler chickens challenged with Clostridium perfringens induced necrotic enteritis. Int. J. Chem. Stud. 6:13–15.

Redweik, G. A. J., Stromberg, Z. R., Van Goor, A., & Mellata, M. (2020). Protection against avian pathogenic Escherichia coli and Salmonella Kentucky exhibited in chickens given both probiotics and live Salmonella vaccine. Poultry Science, 99(2), 752–762. https://doi.org/10.1016/j.psj.2019.10.038

Reid, G., M. E. Sanders, H. R. Gaskins, G. R. Gibson, A. Mercenier, R. Rastall, M. Roberfroid, I. Rowland, C. Cherbut, and T. R. Klaenhammer. 2003. New scientific paradigms for probiotics and prebiotics. J. Clin. Gastroenterol. 37:105–118.

Samanya, M.; Yamauchi, K. Histological alterations of intestinal villi in chickens fed dried Bacillus subtilis var. natto. Comp. Biochem. Physiol. Physiol. 2002, 133, 95-104.

Unno, T., D. Han, J. Jang, K. Widmer, G. Ko, M. J. Sadowsky, and H.- G. Hur. 2011. Genotypic and phenotypic trends in antibiotic resistant pathogenic Escherichia coli isolated from humans and farm animals in South Korea. Microbes Environ. 26:198–204.

Xia X, Meng J, Zhao S, Bodeis-Jones S, Gaines SA, Ayers SL, et al. Identification and antimicrobial resistance of extraintestinal pathogenic Escherichia coli from retail meats. J Food Prot. 2011 Jan; 74(1):38-44.

Yoon, C.; Na, C.S.; Park, J.H.; Han, S.K.; Nam, Y.M.; Kwon, J.T. Effect of feeding multiple probiotics on performance and fecal noxious gas emission in broiler chicks. Kor. J. Poult. Sci. 2004, 3, 229-235.

Zhang, L., Zhang, L., Zhan, X., Zeng, X., Zhou, L., Cao, G., Chen, A., & Yang, C. (2016). Effects of dietary supplementation of probiotic, Clostridium butyricum, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli K88. Journal of Animal Science and Biotechnology, 7(1), 1–9. https://doi.org/10.1186/s40104-016-0061-4

Zulkifli, I.; Abdullah, N.; Azrin, N.M.; Ho, Y.W. Growth performance and immune response of two commercial broiler strains fed diets containing Lactobacillus cultures and oxytetracycline under heat stress conditions. Br. Poult. Sci. 2000, 41, 593-597.

Kim, Y. Bin, Yoon, M. Y., Ha, J. S., Seo, K. W., Noh, E. B., Son, S. H., & Lee, Y. J. (2020). Molecular characterization of avian pathogenic Escherichia coli from broiler chickens with colibacillosis. Poultry Science, 99(2), 1088–1095. https://doi.org/10.1016/j.psj.2019.10.047