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African Journal of Microbiology Research

*Review*

# *Mycoplasma gallisepticum*: An overview

**Ruizhi Yang[1], Xi Lin[3], Huiqi Song[1], Hongmiao Zhou[1], Shuang Li[2], Xuejiao Li[2], Bin Hao[2] and Lianrui Li[1,2,4,5]***

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**The prokaryotic microorganism *Mycoplasma gallisepticum* (MG) belongs to the phylum Firmicutes, the order Mycoplasmatales, the family Mycoplasmataceae, and the genus Mycoplasma. MG parasitizes the respiratory tract of various poultry species, causing a range of symptoms from indistinct features to tracheitis and air sacculitis. Common signs include respiratory tract infection indicators such as coughing, asthma, a runny nose and rales. Both domestic and international reports underscore MG's transmission and infection capacity, signifying its global importance as a source of poultry infection. Further research is imperative to grasp its impact on both farmed and wild animals. This article comprehensively reviews current research progress on MG, encompassing its biological characteristics, infection traits, pathogenic mechanisms, epidemiology, detection methods, treatment, and control measures. It aims to serve as a reference for comprehensive prevention and control strategies against MG in the future.**

**Key words:** *Mycoplasma galliscepticum*, infection characteristics, epidemiological characteristics, detection methods, treatment, vaccine.

## INTRODUCTION

Chickens play a crucial role in the poultry industry, and poultry production can significantly improve the nutritional status of underdeveloped communities and enhance local food security. Furthermore, it plays a crucial role in promoting economic development in impoverished regions (Truong et al., 2021). In recent years, China's poultry farming industry has experienced rapid growth, resulting in a substantial increase in the production of poultry products. This surge has provided local communities with a significant amount of high-quality animal protein, making a vital contribution to supplementing food security (National Bureau of Statistics of China, 2022).

Avian mycoplasma infection is a significant factor that hinders the development of the poultry industry and is widespread globally (Levisohn and Kleven, 2000). In

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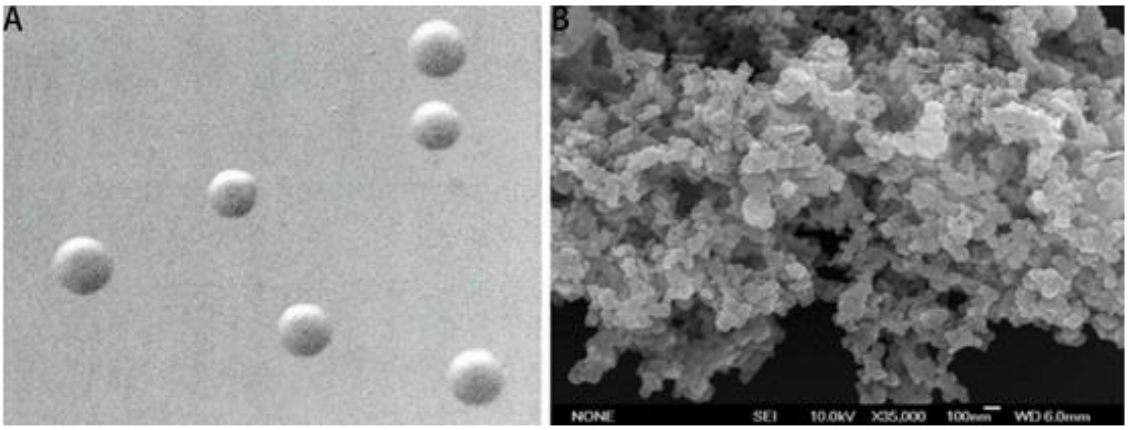


**Figure 1.** *Mycoplasma galliscepticum* colony morphology (A); Microscopic structure of *M. galliscepticum* biofilm (B).

1926, avian mycoplasma infection was first discovered in turkeys, and it was subsequently identified in chickens in 1936 (Yadav et al., 2022). In poultry production, an increasingly concerning issue is the infection of chickens by MG (Dierks et al., 1967). MG is listed by the World Organization for Animal Health as a respiratory pathogen, causing diseases in chickens and turkeys (World Organization for Animal Health, 2019). The Chinese Ministry of Agriculture has also classified it as a Class III animal pathogenic microorganism (Gazette of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2005). MG holds significant clinical and economic implications for the global poultry industry. The economic losses attributed to MG in poultry primarily result in reduced growth rates and decreased feed conversion efficiency in chickens. Additionally, it may lead to decreased egg production and lower hatching rates. It is estimated that MG causes global economic losses exceeding $780 million to the poultry industry each year (Khalifa et al., 2013).

### Basic biological characteristics

MG, which lacks a cell wall, does not have distinct cellular organelles or nuclear-like structures. It is motile and exhibits pleomorphism in size and shape, with the majority being elliptical or round, and diameters ranging from 0.3 to 0.7 μm (Tajima et al., 1979). It displays the typical "fried egg" morphology in colony culture on agar plates (Xie, 2021) and has the ability to form biofilms (Chen, 2012), as shown in Figure 1. The complete genome of MG is approximately 970 kbp-1,000 kbp in length, with a total G+C content of around 31%. It contains minimal genetic information, characteristic of a typical prokaryote (Leigh et al., 2022; Song et al., 2021; Leigh et al., 2019a; Leigh et al., 2019b; Papazisi et al., 2003). MG is a microorganism that can thrive in both aerobic and facultative anaerobic conditions. However, it can only undergo anaerobic growth when acetate is supplied as a hydrogen receptor (Gill, 1962). MG is capable of fermenting glucose, reducing tetrazolium salts, and is sensitive to 1.5% digitonin, while it does not hydrolyze arginine and lacks phosphatase activity (Stallknecht et al., 1982; Marouf et al., 2022a; Cole et al., 1968; Bergey, 1994).

## PATHOLOGICAL FEATURES

### Clinical features

Infections caused by MG can result in chronic respiratory disease (CRD) in chickens and infectious sinusitis (IS) in turkeys (Stipkovits et al., 1996). MG can penetrate the respiratory mucosal barrier, enter the bloodstream, and spread throughout the body. MG can be found in various tissues and organs, such as the respiratory system (air sacs, nasal turbinates, lungs, and sinuses), reproductive tract (oviducts and ovaries), testes, cloaca, as well as mucous membranes of the mammary glands, brain, eyes, gallbladder, and joints (Levisohn and Kleven, 2000; Bencina et al., 1991; Nunoya et al., 1995; Amin and Jordan, 1978). Infections caused by different strains of MG show significant variations in clinical symptoms. Typical symptoms include inflammation of the mucous membranes of the upper respiratory tract and adjacent tissues, coughing, tracheal rales, increased nasal secretions, sinus swelling (common in turkeys), facial swelling, sneezing, increased foamy secretions from the eyes, eyelid swelling, enlarged eyeballs, difficulty breathing, sinusitis, conjunctivitis, air sac lesions, reduced feed intake, and symptoms such as weight loss or even growth stagnation. MG infections can result in acute and chronic diseases affecting multiple sites, often accompanied by a variety of complications (Yadav et al.,

2022; Sun and Yang, 2011). After being infected with MG, males typically experience more severe clinical symptoms than females. These symptoms are most pronounced in the morning and evening. The incidence of the disease is influenced by age, with younger birds being more severely affected than older ones. Additionally, environmental temperature plays a role, as symptoms are more severe and prolonged in colder environments (Stipkovits et al., 1996; Kleven et al., 1998). Turkeys also tend to have a higher incidence of the disease compared to chickens (Jordan and Amin, 1980). It's important to note that the pathogenicity of MG strains in causing respiratory infections in poultry is not necessarily correlated with their pathogenicity for in-ovo infection (Levisohn et al., 1986).

Under natural conditions, the incubation period for MG can vary significantly, ranging from 1 to 21 days or more. In chicken flocks infected through egg transmission, difficulties in hatching or poor chick quality may be observed. Typical clinical symptoms may appear at three to six weeks of age or during the onset of the laying period. However, if adult chickens are individually infected with the pathogen under normal husbandry practices and in good sanitary conditions, or before the onset of related diseases or adverse stress factors, the infection often follows a subclinical or mild course. Lesions typically heal within five to six weeks. MG rarely invades the joints or central nervous system (Fabricant, 1968), and its virulence may decrease as it adapts to the host (Sawicka et al., 2020; Henschen et al., 2023). Maternal antibodies present in the egg can decrease the harmful effects of MG, thereby enhancing the chances of survival for infected embryos (Levisohn et al., 1985). However, instead of manifesting as a single infection, MG often presents as one of several pathogens in a complex of multifactorial diseases. The synergistic interactions among these respiratory pathogens can result in severe clinical symptoms, a high incidence, and increased mortality rates (Naylor et al., 1992; Sid et al., 2015; Abdelaziz et al., 2019).

## Mechanisms of *Mycoplasma galliscepticum* induced pathogenicity

MG is a pathogenic microorganism that presents a significant threat to the poultry industry. MG primarily attaches to the cilia and mucous membranes of respiratory epithelial cells. The close adherence between the mycoplasma and epithelial cells is suggested to be a critical factor in the pathogenic mechanism of the disease.

Hu study (Hu et al., 2021) indicates that MG infection reduces the antioxidant activity in the spleen and thymus tissues, leading to an imbalance in mitochondrial dynamics in these organs. This leads to damage to the structural integrity of the spleen and thymus, ultimately causing oxidative stress and cell apoptosis. The study suggests that these factors may be related to immune impairment, mechanical structural damage, and disease pathogenesis caused by MG infection. Lamas et al. (1969) indicates that the pathogenic mechanism of MG (strain S6) infection primarily involves arteritis. This condition is characterized by fibrinoid necrosis of the arterial wall, proliferation of endothelial cells, and perivascular infiltration of neutrophils, lymphocytes, plasma cells, and other mononuclear cells. Ishfaq et al. (2020a) indicates that MG infection causes inflammation and triggers oxidative stress, leading to a significant reduction in ATPase activity and the mRNA and protein expression levels of energy metabolism-related genes in chicken lungs. Based on these results, it is suggested that MG infection may cause energy metabolism dysfunction, leading to damage in the chicken lungs. Zhang et al., 2020, suggests that MG infection disrupts the structural integrity of the bursa of Fabricius tissue, while also inducing oxidative stress and cell apoptosis. This may be a contributing factor to the compromised immune function of the chicken's bursa of Fabricius. Moreover, MG can evade the host's immune response by entering cells or temporarily suppressing the activity of T cells (Much et al., 2002; Ganapathy et al., 2003).

In some cases, lesions may also result from indirect damage caused by the host's immune and inflammatory responses, rather than the direct impact of the mycoplasma itself. For instance, in the case of MG infection, the respiratory epithelium experiences substantial thickening as a result of mononuclear cell infiltration and hyperplasia of mucous glands. The impaired ciliary activity hinders the effective clearance of mucosal exudates, which is a prominent feature of the infection. This simultaneously leads to increased nasal secretions, tracheal rales, and coughing (Bradbury, 2005; Charlier et al., 1981). Wu et al. (2022) indicates that after MG attachment and colonization on the respiratory epithelium, it can stimulate the mucosal immune system, inducing lymphocyte infiltration and persistent inflammatory responses. This leads to an imbalance in the immune response, resulting in decreased mucosal immunity in the respiratory tract. Additionally, it increases permeability between the epithelial cells of the tracheal mucosa, leading to dysfunction in the mechanical barrier of the chick's mucosa. This reduced protective capacity makes chicks more susceptible to infections from other respiratory pathogens.

## EPIDEMIOLOGICAL FEATURES

The mycoplasma has traditionally been considered to exhibit significant host specificity, but recent research suggests that this theory may need to be re-examined (Béjaoui et al., 2011). MG can easily spread to uninfected birds through direct or indirect contact. Wild animals may

serve as potential hosts and vectors for MG, contributing to the maintenance and transmission of the pathogen (Dhondt et al., 2014). Currently, there are reports indicating that MG can infect a variety of bird species, including crows, pigeons, ducks, parrots, wild chickens, quails, peacocks, geese, and guinea fowl. Among these animals, chickens and turkeys are the most susceptible to infection.

After settling in the upper respiratory tract, MG may progress to a systemic infection. A significant amount of mycoplasmas can be expelled through nasal secretions, breathing, or coughing. Additionally, due to the connection between the chicken abdominal air sacs and the oviduct, MG can infect eggs, leading to transmission to the offspring (Roberts et al., 1967). MG can be transmitted both vertically (via egg transmission) and horizontally (through close contact, contaminated dust particles, infectious aerosols, or respiratory droplets).

## Environmental colonization of *Mycoplasma galliscepticum*

MG demonstrates a certain level of adaptability to the poultry farming environment. The survival time of MG outside the host (in feces, fabric, etc.) ranges from 1 to 14 days, depending on the environmental temperature and the type of material where MG is present. Therefore, barns or materials that are not adequately cleaned and disinfected could also serve as sources of infection. It's worth noting that the longest survival time observed is in eggs. They can survive for 3 weeks in urine sac fluid at 5°C, 4 days in an incubator, and 6 days at room temperature. In the yolk, they can survive for 18 weeks at 37°C or 6 weeks at 20°C. Therefore, eggshell fragments in the incubator are crucial for transmitting infection (Stipkovits et al., 1996). MG can survive for 1 to 5 days in pure water, 4 to 10 days in water with varying nutrient content (1%-10%), and over 21 days in a complete culture medium (Polak-Vogelzang, 1977). MG can survive for 2 to 4 days on feathers, 8 hours to 4 days on cotton, 2 days on rubber and straw, around 8 hours on wood, approximately 4 hours in ears and noses, but it can hardly survive on the skin (Christensen et al., 1994). However, it should also be noted that personnel working with infected chicken flocks are at risk of becoming carriers of MG. However, fresh or frozen poultry meat products intended for human consumption, as well as infertile eggs, are generally not considered to pose a risk of MG infection (Levisohn and Kleven, 2000).

## Vertical transmission of *Mycoplasma galliscepticum*

Like all pathogenic avian mycoplasmas, the primary route of infection transmission and a key consideration for regional trade is the vertical transmission of MG from infected breeder birds to their offspring inside the eggs. In most cases, the infection status of offspring chickens with MG is determined by the infected breeder flock from which they originated (Michiels et al., 2016). In infected hens, MG can replicate in the ovaries and oviducts. During the acute phase, it is likely to lay eggs (Fabricant, 1968), and can be isolated from the yolk membrane of recently laid eggs (Stipkovits et al., 1996). During the incubation process, some of the infected embryos may perish, while others will hatch and introduce MG into the offspring population. Reports indicate the presence of MG in the semen of males, suggesting the potential for transmission through artificial insemination (Bradbury, 2005).

## Horizontal transmission of *Mycoplasma galliscepticum*

In addition to intra-egg infection, the upper respiratory tract, particularly the trachea, is generally considered the preferred site for natural infection by MG (Sprygin et al., 2011). The pathways of host exposure, the infectious dose of MG, and environmental factors such as temperature, humidity, ventilation, dust, and ammonia concentration, as well as the physiological health status of poultry and their breeds, all play a role in influencing the infection and transmission of MG.

Currently, it has been reported that birds infected with MG can carry the infection for their lives. MG may also spread between wild birds and domestic poultry, so we cannot dismiss the potential impact of wildlife on the transmission of MG (Dhondt et al., 2014). However, in poultry flocks raised under favorable farming conditions, many outbreaks of MG appear to be caused by human factors. The transmission of this disease depends on factors such as the poultry population, the number of susceptible individuals, stocking density, and the concentration of ammonia. With an increase in the number of susceptible individuals and a reduction in the distance between each individual, the likelihood of horizontal transmission occurring may increase (McMartin et al., 1987).

The risk of transmission also depends on the number of infectious agents in each individual. Therefore, when the quantity of MG in the respiratory tract reaches its peak, it may be more likely to be transmitted during the acute phase of infection (Soeripto et al., 1989a). Current research indicates that the peak of upper respiratory tract infection with MG occurs approximately 2 weeks after infection, after which the peak starts to decrease (Yagihashi and Tajima, 1986; Kleven, 1981). Exposure to environmental ammonia exacerbates the severity and duration of MG infection, particularly in relation to ammonia concentration (Sato et al., 1973; Kempf et al., 1988). It is worth noting that when MG is present as one of the pathogens in a multifactorial disease complex, it

amplifies the severity of infection and the probability of transmission (Barbour et al., 1997; Zorman et al., 2021; Habte et al., 2022; Tekelemariam et al., 2022). As mentioned above, MG infection involves both horizontal and vertical transmission. However, the mechanisms and pathways, particularly those related to transmission to humans, are still worth further exploration.

## ECONOMIC LOSS STUDY

MG is a financially important infectious disease in the poultry industry. Avian influenza imposes significant economic losses on poultry production by reducing egg production and hatchability, increasing chick mortality, slowing growth rates and feed conversion efficiency, lowering carcass quality, and incurring additional costs for prevention and treatment.

In broiler chickens, MG infection can lead to a 20% to 30% decrease in weight gain, a 10% to 20% reduction in feed conversion efficiency, a 10% to 20% increase in carcass discard during processing, and a 5% to 10% rise in mortality rates. In layer hens, MG infection can result in a 10% to 20% decrease in egg production, with an average reduction of 16 eggs per hen. This effect is especially noticeable in laying hens infected during the peak of egg production, leading to a 5% to 10% increase in embryo mortality rates (Yadav et al., 2022; Stipkovits et al., 1996; Glisson et al., 1984). After being infected with MG, the egg shape index, eggshell thickness, eggshell strength, and eggshell color index of eggs significantly decrease, leading to a negative impact on egg quality (Hu et al., 2022). Furthermore, simultaneous infections, substandard poultry housing, high breeding density, and insufficient sanitation conditions significantly contribute to increased economic losses (Levisohn et al., 2000).

## DETECTION METHODS

Early diagnosis is crucial for preventing the spread of field strains of MG infection. The sampling, transportation, and handling of samples are critical aspects of detecting MG in diagnostics. Swab samples taken from the cloaca, cloacal bursa, and trachea are used to isolate and detect pathogens in live poultry. In deceased poultry, swab samples taken from the trachea, air sacs, and lungs are used to isolate and detect the pathogen. In dead poultry, swab samples taken from the trachea, air sacs, and lungs are used for isolating and detecting the pathogen. The clinical symptoms and pathological morphological changes in the respiratory system are the main features of MG infection. However, diagnosing based solely on clinical or post-mortem results may be confused with other infectious respiratory diseases. Therefore, laboratory confirmation is typically required for the diagnosis of MG infection (Kleven.1998). In recent years, significant progress has been made in the diagnostic methods for MG. These advancements are evident in the regular updates to standard testing procedures, supported by extensive field experience.

### Isolation culture detection methods

The conventional pathogen culture method stands as the "gold standard" for diagnosing MG infections. In this method, 1 ml of sterile deionized water sample containing tracheal swabs is added to modified Frey's broth and cultured for 24 hours at 37°C, 5% $CO_2$, and high relative humidity. Successful growth is indicated by the change in color of the Frey's broth from red to orange-yellow. The positive broth is inoculated onto solid Frey agar medium and cultured for at least 2 weeks at 37°C with 5% $CO_2$ in a humid environment. Colony morphology is examined under an inverted microscope. Identification of MG is then performed using a growth inhibition test with specific antisera (Hamzah et al., 2022; Erno et al., 1967; Huazhong Agricultural University, 2023). The culture medium for MG must contain serum from pigs, horses, or cows, as well as yeast extract, glucose, and other essential nutrients. Additionally, it requires penicillin and thallium acetate as inhibitors to prevent bacterial and fungal growth. However, due to its relatively slow growth (typically taking one to three weeks, or even longer), the cultivation process is labor-intensive and expensive, requiring sterile conditions. Challenges may also arise during cultivation, such as the overgrowth of saprophytic mycoplasmas and other pathogens, or issues with the lack of growth in subcultures. Additionally, this cultivation technique is labor-intensive, expensive, and time-consuming. It requires skilled technicians, and there may be challenges in isolating MG, which restricts its application.

### Molecular biology detection methods

The development of molecular detection techniques holds great potential for the detection of MG, providing valuable tools for the poultry industry to better control mycoplasmosis (Hamzah et al., 2022). In recent years, molecular diagnostic methods with high sensitivity and specificity for detecting MG have been developed. These methods are crucial tools for studying the epidemiology of MG, enabling effective detection of the pathogen in various clinical samples. Results from molecular detection can be obtained within one to two days, which is a significant improvement compared to the usual one to three weeks required for the isolation and identification of MG through conventional culture methods. Samples used for molecular detection are usually pooled, with approximately three to five tracheal swabs per reaction, to increase the sample volume and reduce testing costs.

However, pooling samples may increase the likelihood of substance inhibition from mucus or other tissue fluids, thereby reducing the sensitivity of detection.

### *Polymerase chain reaction detection methods*

The primary purpose of Polymerase Chain Reaction (PCR) is to rapidly replicate specific regions of DNA or RNA for detection (Mullis et al., 1987). PCR is a rapid, sensitive, and specific method commonly used to detect the presence of specific mycoplasma DNA, often replacing traditional culture-based methods. Marouf et al. (2022b) utilized the Mgc2 gene from MG to identify the presence of the pathogen in chicken and turkey farms in Egypt. The study found differences in the rates of isolating MG from various anatomical sites. The highest isolation rate was observed in sinus infraorbitalis aspirates, followed by the lungs, air sacs, and tracheal bifurcation, while the lowest isolation rate was from tracheal swabs.

The study also indicated that MG has the highest prevalence in Egypt during the winter and autumn seasons. Rasoulinezhad S et al. (2017) conducted a study on the detection of MG in turkey farms in Iran, utilizing the 16S rRNA gene and Mgc2 gene. The study found MG in both commercial and free-range turkey farms in Iran, with a higher rate of infection in free-range farms compared to commercial farms. It was also observed that the positivity rate for MG is higher in the arid inland regions of Iran compared to the coastal humid regions. Kang et al. (2023) used multiplex PCR to target four loci (parE, RS03710-rlmB, scpA, and MGF_RS03965) of MG in order to distinguish between three vaccine strains (ts-11, 6/85, and F strains) and wild-type strains of MG. Tan et al. (2014) utilized a PCR reaction combined with reverse transcription polymerase chain reaction (RT-PCR) to target the MG 16S rRNA gene and assess the viability of MG. This method helps to detect and to some extent distinguish between living and dead MG.

### *Real-time fluorescence quantitative PCR*

Real-time quantitative PCR (qPCR) is an accurate, rapid, sensitive, and cost-effective method, making it one of the most effective techniques for gene expression analysis (Harshitha et al., 2021). Galluzzo et al. (2022) conducted a study to detect MG in egg-laying hens in Sicily, Italy, using real-time quantitative PCR. The study revealed an infection rate of 28.6% in commercial chicken flocks and 40.0% in rural free-range chicken flocks. Interestingly, the sampled hens did not exhibit any clinical symptoms, indicating that the infection could spread unnoticed. Ferguson-Noel et al. (2012a) used real-time PCR to study the effect of different swab materials (nylon flocked, cotton, and polyester fibers) on MG sampling. The study indicated that the various swab materials did not have a significant impact on the real-time PCR results for detecting MG.

### *Loop-mediated isothermal amplification*

The loop-mediated isothermal amplification (LAMP) assay has emerged as an affordable and rapid molecular diagnostic technique. In 1998, a Japanese company called Eiken Chemical Co., Ltd. developed the LAMP method (Soroka et al., 2021).

Ehtisham-Ul-Haque first developed the LAMP method for detecting MG using the mgc2 gene sequence (Ehtisham-Ul-Haque et al., 2017). Zhang et al. (2015) analyzed and designed LAMP primers based on the pdhA gene sequence of MG. The research results indicate that real-time LAMP detection methods can be a simple and effective way to detect MG in poultry.

## Serological testing

In recent years, significant advancements have been made in modern immunological detection techniques based on the principle of antigen-antibody binding, due to the rapid development of monoclonal antibody technology. Compared to molecular detection, serological testing requires less time and is more cost-effective. However, using serological testing to determine the infection status of MG may have some limitations. Currently, it has been observed that certain proteins of MG exhibit serological cross-reactivity with proteins from other Mycoplasma species, such as Mycoplasma synoviae (MS) (Yogev et al., 1989). Therefore, cross-reactions may occur in serological tests, which can lead to a lack of specificity or sensitivity. However, greater specificity can at times reduce the test's detection capability. In almost any serological test, there is a possibility of false positive results. Therefore, relying solely on a single detection method is not advisable (Stallknecht et al., 1982). Despite the numerous limitations of serological testing, it continues to be an essential diagnostic tool for detecting MG in countries with prevalent outbreaks, particularly in developing nations or rural areas where there may be a shortage of laboratory equipment and specialized technical personnel. This is due to its affordability and relatively straightforward operation (Yagupsky et al., 2019). Therefore, serological tests can be used initially to screen samples for MG infection, followed by confirmation through culture or molecular detection techniques.

### *Enzyme-linked immunosorbent assay*

The Enzyme-Linked Immunosorbent Assay (ELISA) is a

method that uses color changes resulting from enzyme-linked conjugates and enzyme substrates to demonstrate antigen-antibody reactions. It is used to identify and quantitatively analyze molecules in biological samples by determining their presence and concentration (Coons et al., 1941). Shiferaw J et al. (2022) conducted a study using the indirect ELISA method to detect the seropositivity of MG in commercially raised laying hens on poultry farms in Ethiopia. The overall serum positivity rate for MG infection was found to be 70.65%. The research revealed significant variations in the positivity rate of MG among different chicken breeds, with a higher incidence in Bovans chicken breeds compared to Sasso chicken breeds. Additionally, adult chickens exhibited higher serological positivity rates than young chickens. Muhammad J et al. (2021) conducted ELISA-based testing in the poultry industry in Pakistan and found an overall serum positivity rate of 65% for MG infection. The study revealed variations in the positivity rate of MG among different types of chickens. Layers had the highest positivity rate, followed by breeder chickens, while broilers exhibited the lowest positivity rate. Ali et al. (2015) utilized the ELISA method to detect MG antibodies in commercial laying hens in Bangladesh. The study revealed significant variations in the prevalence of disease among different chicken breeds. The occurrence of MG was found to increase in cold weather and high relative humidity conditions. Furthermore, there was a positive correlation between higher chicken population density and an increased prevalence of MG.

### *Lateral flow immunochromatographic analysis*

Lateral flow assays (LFAs) are paper-based platforms used to detect and quantify analytes in complex mixtures. The sample is placed on the testing device, and results are displayed within 5-30 minutes, making it easy to detect specific analytes in fluids or other samples (Gao et al., 2018; Koczula and Gallotta, 2016).

Elyazeed et al. (2020) conducted a study to develop, assess, and compare LFAs with commercial ELISA kits and PCR methods. The research indicated that lateral flow assays demonstrated lower sensitivity compared to PCR but higher specificity. This suggests that the prepared LFAs can be used as a rapid diagnostic technology for detecting avian MG in Egypt. This marks the initial development of LFAs technology for diagnosing MG infection in Egypt.

### *Serum plate agglutination and hemagglutination inhibition test*

Seurm Plate Agglutination (SPA) and Hemagglutination Inhibition (HI) are known for their simplicity, cost-effectiveness, sensitivity, rapid result determination, and the absence of a need for special equipment. However, these methods often encounter issues with non-specific reactions (Ross et al., 1990; Feberwee rt al., 2005; Bibby et al., 2022).

Branton conducted a study on the impact of different swab materials (artificial silk, calcium alginate) on the sampling of MG, using serum plate agglutination and hemagglutination inhibition tests. The research indicated that the absorbency of various swabs does not affect the recovery and subsequent isolation of MG (Branton et al., 1985). Branton, (1984) conducted a study using serum plate agglutination and hemagglutination inhibition tests, which revealed a higher quantity of MG isolated from cloacal swabs compared to tracheal swabs. The study highlighted that cloacal swabs are easier to manage and less harmful to birds than tracheal swabs. It's important to note that tracheal swabs are susceptible to contamination from feed, especially in birds that have just finished eating or are in the process of eating. Branton et al. (1991) conducted serum plate agglutination and hemagglutination inhibition tests, and found that wiping commercial laying hens with sterile Frey broth-moistened synthetic swabs and wiping them with the same type of dry swabs did not affect the subsequent recovery and isolation of MG.

## PREVENTION AND TREATMENT OPTIONS

Mcmartin (1995) discussed the strategy for controlling infectious diseases in poultry, stating, "From the perspective of strategic disease control, there are only two types of infections: those that can be excluded from the herd and those that cannot be excluded.". The control measures for MG in poultry include three aspects: maintaining a disease-free flock, drug treatment, and vaccination. Therefore, treatment, preventive measures, and other control strategies are crucial. Control measures mainly involve improving hygiene, regularly testing the flock for mycoplasma infection using appropriate methods, and administering suitable drugs for prevention (prophylaxis), treatment, or eradication, or vaccination. The rapid growth of poultry production in various regions worldwide has resulted in the establishment of numerous large, multi-age poultry farms, where a significant number of poultry are typically raised in relatively compact housing areas. In such situations, it is challenging to keep all poultry flocks free from MG infection. Therefore, appropriate medication or vaccination can be used to alleviate clinical symptoms, reduce production losses, or prevent egg transmission.

### Routine prevention program

In theory, the control of MG infection is relatively simple and direct, especially since the pathogenic MG primarily relies on egg transmission. To begin, select MG-free

chicken breeds and raise them in a secure farm environment. Implement a robust monitoring system to promptly identify and eliminate any infected populations. This approach has been successful in China and many other countries and regions worldwide. However, achieving the goal of complete control of MG infection still appears to pose certain challenges (Kleven, 2008).

### *Egg transmission prevention program*

One significant route of MG transmission is through egg transmission. Therefore, interrupting the egg transmission pathway can effectively prevent the spread of MG. Antibiotic treatment: The process involves heating fertile eggs to 37°C and then rapidly immersing them in a solution of erythromycin or tylosin (concentration: 400-1,000 mg/L) that has been pre-cooled to 5°C. This 15-20 minutes process aims to reduce or eliminate the transmission of MG through eggs. Importantly, this egg immersion procedure minimally impacts hatchability rates (Ba, 2014; Hall et al., 1963; Olson et al., 1962; Ortiz et al., 1995). While antibiotics are effective tools for reducing egg transmission, they cannot completely eliminate infection in the flock. Prolonged use of antibiotics may also contribute to the increase in resistance in MG. Therefore, while drugs can effectively prevent the associated economic losses of MG infection, they should not be considered a long-term solution but rather a short-term means to alleviate symptoms or economic impacts. Heating method: The fertilized eggs can be warmed to approximately 46°C for 12-14 hours and then adjusted to the normal incubation temperature (Yu, 2021; Nie, 2020; Yoder, 1970). Simultaneously, research indicates that exposing fertilized eggs to a temperature of 46.5℃ for 20-30 minutes can achieve the same eradication of MG within the eggs. However, this method leads to a decrease in the hatching rate of fertile eggs by approximately 8% to 12% (Ma, 2018). Additionally, before storing the fertile eggs in the repository, fumigation with formaldehyde or potassium permanganate can be used (42 mL of formaldehyde +21g of potassium permanganate, for 30 minutes) to achieve further reduction of MG. Additionally, before storing the fertile eggs in the repository, fumigation with formaldehyde or potassium permanganate can be used (42 mL of formaldehyde +21g of potassium permanganate, for 30 minutes) to achieve further reduction of MG (Huang et al., 2022; Lai et al., 2022). At the same time, in regional trade, breeding facilities should offer assurance to buyers of breeding chickens or hatching eggs to guarantee that no infection has occurred, in order to prevent the spread of the disease (Bennett et al., 2013).

### *Environmental transmission prevention program*

Another means of transmitting MG is through environmental horizontal transmission. Preventing the horizontal spread of MG in the environment can be effectively achieved by implementing stringent daily management practices on poultry farms. Breeding units should implement preventive measures in various aspects, including sourcing poultry, transferring poultry, disposing of dead poultry, removing feces, and managing personnel and equipment involved in these activities (Halvorson, 2011).

Free-range chickens primarily live outdoors, which increases the risk of contact with wild animals compared to confined chicken flocks. Therefore, efforts should be made to minimize contact with wild animals. Chickens confined on the farm should undergo strict breeding procedures, preferably adopting a self-breeding and self-sustaining rearing model to avoid introducing sources of infection. If an introduction is necessary, chicks must be purchased from breeder farms that are free from mycoplasma infection. Upon arrival, the newly introduced chickens should undergo testing and be placed in isolation for observation. Only after confirming their health and absence of infection should they be allowed to mix with the existing flock. Improving the farm's management is essential. Implementing strict all-in, all-out farming practices is essential. This involves completely segregating broilers, pullets, breeders, and hatchery facilities. Moreover, thorough cleaning and disinfection should be conducted after the removal of each batch of chickens. MG has low resistance to the environment, so regular disinfectants or ultraviolet irradiation can be effective for disinfecting the environment and equipment (Ghosh et al., 1977). During the rearing of chicken flocks, it is important to maintain cleanliness in the poultry house by cleaning it regularly. Spraying cottonseed oil or using hot spray disinfectants can reduce the amount of dust and microorganisms in the air (Griffin et al., 1970; Adell et al., 2015). The farm should keep the chicken house clean and hygienic, maintain proper ventilation to ensure fresh air inside the house, regulate stocking density appropriately, prevent excessive temperature fluctuations and humidity, and eliminate various stress factors as much as possible. It is crucial to ensure that the ammonia levels in the air inside the chicken house are within normal limits. Spraying edible vinegar can help reduce the ammonia content in the chicken house (Anderson et al., 1968).

## Vaccine prevention and control plan

Poultry infected with MG can cause severe pathological changes and significant economic losses, particularly in commercial egg-laying hens and broiler breeder flocks. The use of vaccines is a primary method for preventing and controlling MG, leading to a reduction in production losses (Leigh et al., 2019). Adler first proposed vaccinating poultry against MG in 1960 (Asler et al., 1960). So far, both live attenuated vaccines (such as the

F strain, ts-11 strain, and 6/85 strain) and inactivated vaccines (such as oil-emulsion vaccines) have been commercially used (Ishfaq et al., 2020b). Each vaccine strain differs significantly from other vaccine strains or fully pathogenic MG in terms of biological characteristics, including infectivity, pathogenicity, persistence, and immune response (Branton et al., 2002). Poultry farms should consider various factors when choosing a vaccine type and strain, including cost, administration route, virulence of local prevalent strains of MG, the likelihood of inadvertent exposure to susceptible neighboring flocks, and the potential for reversion to virulence. To minimize the risk of vaccine spread to other flocks, any live MG vaccine should demonstrate low infectivity and have easily distinguishable markers between the vaccine strain and the wild type (Whithear, 1996).

### *Mycoplasma galliscepticum live attenuated vaccine*

Live MG vaccine strains must replicate in poultry to elicit protective immune responses that last, without causing disease or spreading to other susceptible birds. The virulence of MG strains is inversely correlated with their capacity to stimulate protective immune responses. Strains that are completely avirulent are unlikely to stimulate the immune system (Adler and da Silva, 1970; Soeripto et al., 1989b; Levisohn, 1984). Currently, there are five primary attenuated live vaccines for MG, which include strains 6/85 strain, ts-11 strain, F strain, K strain, and ts-304 strain. These vaccine strains have effectively reduced losses associated with MG infection. However, they vary in terms of the protection they provide, pathogenicity, and transmissibility.

***Mycoplasma galliscepticum* F vaccine strain:** The MG F strain was first isolated by (Yamamoto and Adler, 1956; Levisohn and Kleven, 1981). The F strain vaccine of MG has lower toxicity compared to many wild strains, and it also shows a reduced rate of transmission among birds, which decreases its pathogenicity. Additionally, this vaccine strain can supplant field strains over a specific period (Vance et al., 2008a; Purswell et al., 2012; Kleven et al., 1998). In commercial poultry farms, chickens are usually vaccinated with live MG vaccine strains when they are between 8 and 10 weeks of age. Vaccination after 18 weeks of age is less effective and is associated with reduced egg production, with an average decrease of 10.3 eggs (Carpenter et al., 1981). Furthermore, research suggests that vaccination with the F strain may lead to changes in egg production in commercial layers, which could be linked to alterations in egg yolk composition (Burnham et al., 2003). The F strain vaccine is stored by freezing or freeze-drying (Lin et al., 1984a). The F strain vaccine can be administered through a mist, eye drops, or in drinking water. Reports suggest that administering the vaccine through drinking water, rather than misting, can prevent significant production losses, resulting in an average increase of 4.7 eggs (Carpenter et al., 1981). At the same time, eye drop vaccination is significantly more effective than intranasal drop administration (Leigh et al., 2018). However, it is worth noting that, in comparison to other vaccination methods, administering the vaccine through a spray can lead to a quicker immune response (Purswell et al., 2010; Branton et al., 2005).

When comparing the performance of chicken flocks vaccinated with the F strain and those infected with wild-type MG, the flocks vaccinated with the F strain produce an additional 7.0 eggs per hen per year (Carpenter et al., 1981). However, the egg production of broiler breeders vaccinated with the F strain is significantly higher than that of broilers not vaccinated with the F strain (Liu et al., 2013; Vance et al., 2008b). Compared to the group without the F strain, the hens vaccinated with the F strain vaccine experienced a 3-day delay in the onset of egg production (Carpenter et al., 1981).

Kleven (1985) investigated the stability of the MG F strain at various temperatures and in different buffering agents. The research indicates that the MG F strain remains stable for 24 hours in various buffering agents at both 22°C and 4°C environments. This conclusion is consistent with the study by Leigh et al. (2008), which indicates that phosphate-buffered saline (PBS) prevents the loss of live vaccine viability. In distilled water, the F strain vaccine remains stable at 37°C for up to 4 hours. A decrease in titer is observed after 8 hours, and by 24 hours, the culture is no longer viable. Research indicates that the F strain can be stored for an extended period at -60°C, while at -20°C, the titer rapidly decreases. It is important to note that the inclusion of cryoprotectants (10% glycerol or 8% dimethyl sulfoxide) or freeze-drying stabilizers (12% sucrose, 7.5% glucose, 8% skim milk, 1% bovine serum albumin) does not help in preserving the viability of the mycoplasma during the freezing or freeze-drying process and subsequent storage (Lin and Kleven, 1982). The results indicate that storing the F strain in a freeze-dried state at -60°C is a cost-effective and straightforward storage method.

The F strain vaccine, despite its lower pathogenicity, has been reported to spread horizontally and cause infections in both chickens and turkeys, leading to adverse effects. Khalifa et al. (2014) reported that in Egypt, the F strain did not replace the wild strain and was able to infect chicken populations that had not been vaccinated. There are reports indicating that concurrent administration of an F strain aerosol vaccine, along with a combined Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) vaccine, can lead to air sac damage (Rodriguez et al., 1980). Furthermore, the F-strain exhibits high pathogenicity in young turkeys (Lin and Kleven, 1984b; Lin and Kleven, 1982b). Simmons et al. (1988) noted that when broiler breeders housed in conventional chicken coops were exposed to high

temperatures, the rectal temperature of the F strain vaccinated hens was significantly higher than that of the unvaccinated control group. This suggests that the F strain may contribute to the higher mortality rate caused by heat stress in broiler breeders.

***Mycoplasma galliscepticum* 6/85 vaccine strain:** Currently, the origin of the MG 6/85 vaccine strain is not well-defined (Whithear, 1996). However, based on restriction enzyme analysis and protein analysis, it is indicated that the MG 6/85 vaccine strain is closely related to the MG S6 strain. The recommended method of administration for the MG 6/85 vaccine strain is through aerosol inoculation (Evans et al., 1992). The 6/85 vaccine strain elicits a less robust protective immune response compared to the F strain. It also exhibits lower virulence and infectivity, resulting in no embryo or post-hatch chick mortality (Alqhtani et al., 2023). The 6/85 vaccine strain can be provided as a freeze-dried product for administration via spray vaccination (Whithear et al., 1990a). The antibody response in chickens following spray vaccination is initially weak but increases over time (Noormohammadi et al., 2019). Leigh et al. (2010) noted that chickens vaccinated with the 6/85 vaccine strain can be revaccinated with the F strain to enhance protection against the vaccine strain and reduce adverse reactions associated with direct F strain vaccine inoculation.

***Mycoplasma galliscepticum* ts-11 vaccine strain:** The MG ts-11 vaccine strain was developed by exposing the field-isolated strain (strain 80083) of extreme virulence from Australia to 100 µg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and selecting it through low-passage cultivation (Whithear, 1996). The strain was ultimately selected because it displayed a temperature-sensitive (ts+) phenotype. This indicates that the ts-11 vaccine is temperature-sensitive, exhibiting robust growth at 33°C but diminished growth at 39.5°C (Whithear et al., 1990b).

Compared to the F strain, the ts-11 vaccine strain exhibits lower virulence and infectivity (Abd-el-Motelib and Kleven, 1993), resulting in a somewhat milder but generally effective long-term protective immunity (Jacob et al., 2014). The ts-11 vaccine strain is stored by freezing and is administered to chicken flocks aged 2 to 16 weeks (Gaunson et al., 2006). The ts-11 vaccine strain is administered through eye drops and can be used in combination with other respiratory disease vaccines. The initial systemic antibody response after vaccination is robust, but it gradually diminishes over time. However, the vaccine strain can be isolated from the upper respiratory tract throughout the poultry's life cycle, leading to long-term immunity (Whithear, 1996). Research on the blood characteristics of commercial laying hens suggests that the combination of the ts-11 vaccine strain and the F strain may offer improved protection against wild strains of MG. It might also help to overcome some adverse reactions associated with using the F strain alone (Peebles et al., 2009).

***Mycoplasma galliscepticum* K vaccine strain:** The MG K vaccine strain (K5831) was developed by the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia. It is a naturally occurring non-pathogenic strain of MG that offers protection to the respiratory and reproductive systems, preventing infection from field strains (Ferguson-Noel et al., 2015). The K vaccine strain is administered via the aerosol method. The horizontal and vertical transmission rates of the K vaccine strain are both low. After vaccination with the K strain, it can persist in the upper respiratory tract of vaccinated birds for up to 5 months. Additionally, the K vaccine strain has low virulence and does not cause significant damage to the air sacs or trachea in the host chickens (Ferguson-Noel et al., 2012b).

***Mycoplasma galliscepticum* ts-304 vaccine strain:** The ts-304 strain of MG is a variant of the ts-11 strain. The ts-304 vaccine strain contains a complete and fully functional gapA gene. This vaccine strain is capable of more effectively colonizing the trachea and offers protection to the host against infection by wild-type strains. The ts-304 vaccine strain is usually preserved through freeze-drying and administered via the ocular route (Kanci et al., 2020a). Compared to the ts-11 strain, the ts-304 vaccine strain has lower virulence and infectivity, a longer duration of immunity, and appears to have higher potential as a candidate vaccine (Shil et al., 2011; Kanci et al., 2020b; Kulappu et al., 2021a; Kulappu et al., 2021b). It's noteworthy that reports indicate immune suppression caused by infections with Chicken Anemia Virus (CAV) and Infectious Bursal Disease Virus (IBDV) can interfere with the protective effects of the ts-304 vaccine strain in chickens (Kulappu et al., 2021c. Therefore, when using live vaccines to eliminate Avian Mycoplasma, initially inoculate with the F strain to eliminate potentially existing wild strains. After the F strain replaces the original field strain (which is estimated to take at least one production cycle), you can switch to less virulent, with milder respiratory reactions and safer vaccine strains like 6/85 strain or ts-11 strain (Kleven et al., 1998).

### *Mycoplasma galliscepticum killed vaccines*

Inactivated vaccines (killed vaccines) are effective in preventing respiratory diseases in chickens and have been shown to help reduce transmission and production losses. Moreover, they are considered safer compared to traditional live vaccines (Muofaq et al., 2023; Talkington and Kleven, 1985). Marouf et al. (2022a) developed an inactivated pentavalent vaccine targeting Salmonella typhimurium (ST), Salmonella enteritidis (SE), Salmonella

Kentucky (SK), MG, and MS. The inactivated vaccine was found to provide good protection against Salmonella infection and avian mycoplasma disease. Currently, a bivalent inactivated vaccine for chicken mycoplasma and chicken synovial bursitis mycoplasma has been produced, which can simultaneously prevent both pathogens, reducing the workload of vaccine administration (Qingdao Yibang Biotechnology Co., Ltd., 2016; Nanjing Tech-Bank Bio-industry Co., Ltd., 2015). Hussein Ael-D (Hussein et al., 2007) demonstrated that 2-bromoethylamine inactivated vaccine and saponin inactivated vaccine provide protection similar to the F strain, while formalin-inactivated vaccine offers less protection. It was noted that inactivated vaccines can induce a certain degree of protective immunity in chickens.

Inactivated vaccines are typically used in combination with adjuvants to enhance their ability to induce immune responses (immunogenicity), administered through subcutaneous or intramuscular injection (Pollard et al., 2021). The β-propiolactone-inactivated MG Tween-80 oil emulsion vaccine can reduce the decrease in egg production caused by MG infection and provide protection against respiratory infections caused by MG (Yoder and Hopkins, 1985). Limsatanun et al. (2018) suggested that chitosan could be an effective mucosal adjuvant, enhancing the efficacy of MG vaccine and reducing the pathological lesions induced by MG.

However, while inactivated vaccines can temporarily control MG infection (e.g., prevent egg production loss, respiratory lesions, and egg transmission), they cannot prevent infection. Additionally, they come with high labor costs, requiring the handling of individual birds, and may cause localized adverse reactions at injection sites (Liu et al., 2013; Kleven et al., 1984). Therefore, Hein (2004) pointed out that attenuated live vaccines are a better choice for preventing MG infection compared to inactivated vaccines.

### *Mycoplasma galliscepticum genetic engineering vaccine*

The traditional vaccine development process is lengthy and expensive. Additionally, existing attenuated live vaccines and inactivated vaccines demonstrate pathogenicity and adverse effects. Therefore, there is a need to design and develop more effective and cost-efficient novel recombinant vaccines. The advancement in immunoinformatics tools enables the rapid design and development of more targeted vaccines.

Advancements in bioinformatics and immunoinformatics now enable the efficient screening and validation of proteins, which facilitates the identification of potential vaccine targets. Mugunthan (Mugunthan and Harish, 2021a; Mugunthan et al., 2021b) utilized immunoinformatics strategies to develop multi-epitope candidate vaccines for MG. This involved utilizing potential T-cell and B-cell binding epitopes, as well as cell adhesion proteins of MG. Lu et al. (2016) conducted a bioinformatics analysis of the mucosal immune antigen Vlh A4.12 protein from the MG HS strain. The analysis revealed the presence of multiple B-cell and T-cell antigenic sites on this protein, laying the groundwork for the development of MG peptide vaccines. Feng et al. (2013) utilized the reverse genetics system of the Newcastle disease virus (NDV) LaSota low-virulence vaccine strain to create a recombinant virus that expresses the MG TM1 protein, known as rLa-TM1. The final study indicated that the recombinant virus rLa-TM1 could serve as a potential vaccine candidate for MG as a vector vaccine. Vectormune FP-MG is a genetically engineered live fowlpox virus vaccine for use in chickens and turkeys. Reports indicate that vaccination with Vectormune FP-MG in chickens is safe, with no observed adverse reactions to the vaccine and limited horizontal transmission capability. This suggests that the vaccine is effective in preventing both avian pox virus and MG infections in chicken flocks (Zhang et al., 2010; Leigh et al., 2013). However, Ferguson-Noel et al. (2012c) pointed out that the protective effect of the recombinant avian pox virus vaccine in chickens is not as effective as that of inactivated vaccines and F strain vaccines.

The utilization of recombinant vaccine technology can offer a safe and effective solution for avian mycoplasma vaccination, but additional exploration and validation are still required. Additionally, strong support from both businesses and governments is required to increase investment in research related to the development of vaccines for preventing avian mycoplasma infection.

### Drug prevention and treatment plan

Currently, drugs have been utilized to treat respiratory infections, decrease egg transmission, and boost egg production in commercial laying hens following avian mycoplasma infection (Nolan et al., 2000). MG is susceptible to various classes of antibiotics, including macrolides, chloramphenicol, tetracyclines, fluoroquinolones, and others. However, due to the absence of a cell wall, MG inherently resists beta-lactam antibiotics such as penicillin and cephalosporin (Gautier-Bouchardon, 2018). Therefore, finding effective antimicrobial drugs is crucial for the clinical treatment of MG infections. (Barbour et al., 1998; Stanley et al., 2001) demonstrates that enrofloxacin can effectively treat infections caused by MG. However, Reinhardt AK et al. (2005) reported that oral administration of enrofloxacin suppresses the pathogenic effects of MG without inducing resistance, but it is unable to completely eradicate it. Kempf et al. (1998) utilized enrofloxacin and difloxacin to treat chickens infected with MG. The research found that treatment with 7.5 mg/kg of difloxacin

for 5 days effectively treated pathogenic MG infection. Additionally, the effectiveness of 10 mg/kg of difloxacin and 10 mg/kg of enrofloxacin was similar in treating respiratory symptoms. Tanner et al. (1993) and Kempf et al. (1992) both used danofloxacin and tilmicosin to treat chickens infected with MG. The research found that the percentage of chickens testing positive in the danofloxacin treatment group was significantly lower than that in the tilmicosin treatment group. The occurrence of airsacculitis in poultry treated with danofloxacin was also significantly lower than in poultry treated with tilmicosin.

Ultimately, danofloxacin was found to be more effective than tilmicosin in preventing airsacculitis, reducing the frequency of MG re-isolation, and preventing seroconversion in surviving birds. Baughn CO's study (Baughn et al., 1978) indicates that the addition of tylosin to the drinking water of turkeys can effectively prevent and treat symptoms of air sacculitis caused by MG. The research also suggests that both tylosin and tilmicosin are equally effective in preventing air sacculitis, but tylosin exhibits twice the activity of tilmicosin in treating infections. Stipkovits et al. (1992) indicates that the simultaneous addition of salinomycin and tylosin to the feed can significantly improve the mortality rate, clinical symptoms, and feed conversion efficiency of chickens infected with MG. Hamdy AH's study (Hamdy, 1969) found that for chickens co-infected with MG and Escherichia coli, the combination of lincomycin and spectinomycin (at a ratio of 1:2) added at 2g per 3.78 liters of water for continuous treatment over 10 days or 3g per 3.78 liters of water for continuous treatment over 7 days can reduce mortality, improve weight gain, and decrease the incidence of pericarditis and perihepatitis. However, if only one drug is used for treatment, it may reduce mortality, but it may not lead to improvements in weight or pathological conditions. Inglis et al. (1964) used tylosin and erythromycin to treat MG-infected turkeys. The research showed that tylosin was more effective than erythromycin in treating infections caused by MG. However, it is worth noting that in vitro experiments indicate that the effectiveness of tylosin against MG is lower than that of erythromycin. Garmyn et al. (2017) indicates that the administration of 12.5/37.5 mg/kg of tilmicosin/tilmicosin for 5 days significantly reduces the quantity of MG in the respiratory tract. It also reduces the severity of lesions in the respiratory system, decreases visible damage in respiratory organs, and promotes weight gain. The combination of tilmicosin and tylosin can reduce the tilmicosin dosage. Timms et al. (1989) treated chickens infected with MG by incorporating tylosin into the feed.

The study found that this approach effectively reduced clinical symptoms and lowered the mortality rate. Garmyn et al. (2019) and Kempf et al. (1997) found that treating chickens infected with MG with doses ranging from 20-300 mg/L of tilmicosin for 5 days significantly reduces growth losses caused by MG infection and decreases the incidence of airsacculitis and peritonitis lesions. Ding H et al. (2013) pointed out that administering a 5 mg/kg dose of marbofloxacin orally every 24 hours can successfully treat chickens infected with MG and Escherichia coli. Glisson et al. (1989) used two different concentrations of tylosin to treat chickens infected with MG. The research indicates that tylosin can effectively control airsacculitis caused by MG. It was also noted that a higher concentration of tylosin is effective in preventing the development of airsacculitis. Furthermore, poultry farmers should also take into account the relationship between drug cost and efficacy. However, chickens infected with MG and treated with tylosin not only show effectiveness against MG but also undergo significant changes in lung microbial communities. When treating MG infections, we need to consider not only the effects of tylosin but also its impact on the host's microbial community (Huang et al., 2021).

The widespread use of antibiotics for preventing and treating MG has resulted in the continuous development of antibiotic resistance due to misuse and improper treatment regimens. Traditional herbs and their primary extracts are considered potential sources of new antibacterial agents. Currently, it has been reported that a series of compounds extracted from traditional herbs can prevent and treat infections caused by avian mycoplasma. Glycyrrhizic Acid has been found to effectively inhibit the proliferation and adhesion of avian mycoplasma, reducing the morbidity and mortality of broilers infected with avian mycoplasma. Treatment with glycyrrhizic acid can significantly improve the production performance of chickens and mitigate damage caused by avian mycoplasma to organs such as air sacs, immune organs, trachea, liver, and heart. At the same time, it is noted that the proper dosage of glycyrrhizic acid treatment (100 mg/kg per day) has no significant adverse effects on broiler chickens (Hu et al., 2022). Baicalin, an active compound found in Scutellaria baicalensis, shows therapeutic potential for chickens infected with MG. Baicalin can alleviate pulmonary pathological changes caused by MG infection, reduce oxidative stress and cell apoptosis, and protect the thymus from structural and functional damage induced by MG infection (Li et al., 2019; Zou et al., 2021; Bao et al., 2021; Chen et al., 2023).

Andrographolide can effectively inhibit the proliferation and adhesion of MG. Furthermore, andrographolide can reduce the morbidity and mortality rates in chickens infected with MG. It can also enhance chicken productivity and alleviate damage to organs such as the air sacs, immune system, trachea, lungs, liver, and heart caused by MG infection (Luo et al., 2023). Chlorogenic acid, extracted from honeysuckle, has been shown to increase the live body weight and reduce pathological damage, making it an alternative therapeutic option for broilers infected with MG (Müştak et al., 2015). Quercetin, an active compound in the traditional herbal

medicine *Ephedra sinica*, has been discovered to effectively inhibit inflammatory damage and oxidative stress caused by MG infection (Wang et al., 2023a). The use of puerarin has been shown to effectively inhibit the inflammatory response and apoptosis induced by MG, thereby protecting the lungs from damage caused by the infection. Research suggests that puerarin may be a potential anti-inflammatory agent that can help chickens resist MG infection (Niu et al., 2020). Luteolin is a natural flavonoid compound known for its exceptional antiviral, antibacterial, immunomodulatory, and anti-inflammatory pharmacological properties. By inhibiting the IL-17/NF-kB pathway, luteolin effectively suppresses MG colonization, thereby alleviating the decline in production performance, inflammatory response, and immune damage caused by MG (Wang et al., 2023b).

## CONCLUSION

MG is a significant pathogen that causes respiratory diseases in poultry, including certain wild bird species. It continues to pose a significant threat to the health and development of the poultry farming industry. The MG vaccines and testing methods currently in use in the market are still imperfect and may have some issues to varying degrees. With the advancements in modern biology, we now have the opportunity to delve deeper into the biological characteristics and genetic variations of MG. This lays a crucial foundation for the development of vaccines and innovative diagnostic tools. Simultaneously exploring the epidemiological patterns, infection mechanisms, and immune evasion strategies of MG will contribute to the development of more comprehensive poultry health management plans and disease prevention strategies. These efforts will ultimately contribute to preventing and controlling diseases caused by MG, thereby safeguarding the health of poultry and promoting the sustainable development of the poultry industry.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Characterization of novel potential probiotic strains of lactic acid bacteria from rat faeces

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**This study aimed to isolate and characterise novel probiotic strains from the faeces of healthy albino Wistar rats. Lactic acid bacteria were isolated on MRS agar, and their probiotic properties were assessed through *in vitro* tests, including tolerance to simulated gastrointestinal juices, auto-aggregation assays, and antimicrobial activity. The antibiotic susceptibility and haemolysis tests were performed to assess the safety of the isolates. Isolates with probiotic potential were selected and identified by 16S rRNA gene sequencing. Five strains, R11, R21, R52, R71 and R81, were Gram-positive and catalase-negative, and they were able to survive the simulated digestive conditions with digestive enzymes and 1% bile salts and could auto-aggregate. All the strains inhibited the growth of indicator pathogens. Additionally, all five strains did not exhibit haemolytic activity and were sensitive to most test antibiotics.These five strains were identified as *Enterococcus faecalis* (R11), *Enterococcus hirae* (R21), *Lactococcus garvieae* (R71), *Lactococcus garvieae* (R52), and *Enterococcus faecalis* (R81). These strains hold potential as probiotic candidates, and further *in vivo* studies are necessary to evaluate their safety and establish putative health benefits.**

**Key words:** Probiotic, *Lactococcus*, *Enterococcus*, rat faeces, haemolytic activity.

## INTRODUCTION

The widespread use of antibiotics has resulted in the emergence of multidrug-resistant microbes, which pose a significant threat to human health. Consequently, there is increasing interest in exploring probiotics and related products as potential antibiotics alternatives (Bazireh et al., 2020). Probiotics are living microorganisms, including bacteria, yeasts, and moulds, that confer benefit to the host's health when consumed in sufficient amounts (Byakika et al., 2019). These microorganisms are crucial in providing significant health benefits to their host and are generally necessary for human health and nutritional needs. Probiotic bacteria, such as *Lactobacillus*, *Pediococcus,* *Bifidobacterium*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* have been identified in fermented foods as well as in the gastrointestinal tracts of animals and humans (Ayivi et al.,

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2020).

Putative probiotic strains require thorough *in vitro* testing to evaluate their safety and functional properties. These evaluations include survival under gastrointestinal conditions, acid and bile salt tolerance, antibacterial activity, antibiotic susceptibility, and haemolytic activity (Byakika et al., 2019). Lactic acid bacteria (LAB) are a prominent group of probiotic bacteria commonly used in various applications (Shehata et al., 2016). LAB are Gram-positive, catalase-negative, facultative anaerobic bacteria and can be either cocci or rod-shaped. During carbohydrate metabolism, LAB produce lactic acid as the primary fermentation by-product (Quinto et al., 2014). LAB are safe for human consumption due to their long history of use in the production of fermented foods, and are generally recognized as safe (GRAS) microorganisms (Shehata et al., 2016). Probiotic Lactic Acid Bacteria can confer numerous health benefits to the host, including anti-diabetic activity (Rittiphairoj et al., 2021; Wang et al., 2020), prevention of allergies (Lei et al., 2021), cholesterol-lowering effects (Gil-Rodríguez and Beresford, 2021), protection against pathogens (Barcenilla et al., 2022), immunomodulatory attributes (Shahbazi et al., 2021), and disease risk reduction (Paiva et al., 2020). LAB are therefore important in the development of functional foods and dietary supplements that promote health and prevent diseases.

Probiotics derived from the intestinal microbiota of both humans and animals exhibit distinct characteristics when compared with probiotics sourced from dairy products. One of the most prominent attributes of non-dairy probiotics is their adhesion properties. Such intestinal isolates often display a greater degree of adhesion activity than dairy isolates (Sornplang and Piyadeatsoontorn, 2016). Probiotic strains should be isolated from the same species as their intended host to elicit appropriate host-specific responses. These probiotics showed a higher likelihood of colonising and persisting in the gastrointestinal tract, thus promoting the growth of helpful microorganisms. Consequently, their usage is considered acceptable from an ethical point of view and potentially more effective than their exogeneous counterparts (Gopal and Dhanasekaran, 2021). Probiotic strains not isolated from their intended host may exhibit transient efficacy as they might not colonise the host gut for extended period (Kort, 2014).While rats are commonly used as animal models in probiotic research, most of these studies do not utilise probiotic strains sourced from rats. Moreover, there is a lack of research on isolating novel probiotic strains from rats, leading to a dearth of commercially available rat-derived probiotics for use in rat model studies. Jena et al. (2013) isolated and characterised probiotic lactic acid bacteria from the rat faecal microbiota. However, their study was limited only to lactobacilli. This research study aimed to isolate and characterise novel potential probiotic strains of lactic acid bacteria from albino Wistar rat faeces.

## MATERIALS AND METHODS

### Animals and ethical considerations

Ten eight-week-old male Wistar rats weighing 203 to 294 g, were procured from the Laboratory of Animal Production Unit of the University of Nairobi, Nairobi, Kenya. A veterinarian examined the rats before housing them in the Small Animal Facility for Research and Innovation (SAFARI) at the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya, under a 12 h light/12 h dark cycle at a temperature of 25°C and humidity of 50%. They had access to autoclaved food (chow pellets) and water *ad libitum*. Prior to the commencement of the experiment, the rats were acclimatized for ten days. The study was approved by the JKUAT Institutional Scientific and Ethical Review Committee (approval number JKU/ISERC/02316/1043).

### Isolation and identification of lactic acid bacteria

The animals were separately placed in pre-disinfected cages and left to defecate normally. Two pellets of fresh faecal samples were aseptically collected from healthy rats in the morning and put in sterile 1.5-ml Eppendorf tubes. The samples were serially diluted ten-fold (down to a $10^{-7}$ dilution) in phosphate-buffered saline (PBS). To only select acid-tolerant lactic acid bacteria isolates, each dilution was inoculated in acidified (pH 2.5) de Man Rogosa and Sharpe (MRS) broth (HiMedia Ltd., Mumbai, India, Cat. M369) for 3 h. Subsequently, appropriate dilutions were plated on MRS agar (HiMedia Ltd., Mumbai, India, Cat. M641) supplemented with 0.5% (w/v) of calcium carbonate and incubated anaerobically at 37°C for 48 h (Gupta et al., 2023; Jena et al., 2013). Potential LAB isolates were identified as round, white to cream-coloured colonies with clear halos. The selected colonies were purified on MRS agar by streaking, and subsequently Gram staining and catalase test were performed following the protocol outlined by Ngene et al. (2019). Gram-positive, cocci in shape, and catalase-negative isolates were selected and stored in MRS broth containing 20% glycerol for further analysis.

### Survival in simulated gastrointestinal conditions

The ability of the selected isolates to withstand the gastrointestinal conditions was assessed following the methodology outlined by Saboori et al. (2022) and Celiberto et al. (2018), with minor adjustments. Overnight cultures of the selected isolates were centrifuged at 6000 × g for 10 min at 4°C. The pellets were washed with PBS (pH 6.5) and resuspended in 3 mL of the same buffer. A 1 mL aliquot of bacterial culture suspension ($10^{-9}$ CFU/mL) was then added in 9 mL of simulated gastric fluid containing NaCl 125 mM, $NaHCO_3$ 45 mM, KCl 7 mM, and 3 g/L of pepsin from porcine (Solarbio Ltd., Beijing, China) at pH 2.5. The suspensions were incubated at 37°C (150 rpm) for 3 h and then centrifuged at 3000 × g for 10 min. The supernatants were discarded. These pellets were washed again three times with PBS and resuspended in 9 mL of simulated intestinal fluid (pH = 8.0), containing bile salt (Oxgall Powder, Sigma Aldrich, USA) 10 g/L and pancreatin (Solarbio Ltd., Beijing, China) 1 g/L. The suspensions were incubated at 37°C for another 3 h to complete the 6-h gastrointestinal phase. The number of viable bacteria was counted and expressed as log CFU/mL, and the survival rates of bacteria (in percentage) were calculated using the following formula:

$$\text{Survival rate (\%)} = \frac{\text{CFU count after assay (6h)}}{\text{CFU count before assay (0h)}} \times 100$$

### Auto-aggregation assay

The ability of the LAB isolates to auto-aggregate was assessed by following the method described by Bazireh et al. (2020), with minor adjustments. Briefly, overnight cultures of the LAB isolates were centrifuged at 6000 × g for 10 min at 4°C. The pellets were washed three times with PBS and resuspended in 9 mL of the same buffer. The initial absorbance was immediately measured at 600 nm (Ao). The mixture was then incubated at 37°C, and the absorbance was measured again at 600 nm (At) at different time intervals (6 and 12 h). The auto-aggregation ability expressed in percentages (%) was calculated using the following formula (Pessoa et al., 2017):

$$\text{Auto-aggregation}(\%) = [(Ao - At)/Ao] \times 100$$

### Antimicrobial activity

The antimicrobial activity of the isolates was assessed *in vitro* following to the protocol outlined by Jena et al. (2013) with minor adjustments. Initially, LAB isolates were cultured in MRS broth overnight and subsequently centrifuged at 6,000 × g for 10 min at 4°C. The resulting supernatants were neutralised to pH 7 using 5 N NaOH and filtered through a 0.22 µm filter for sterilisation. Wells with a diameter of 7.8 mm were created in agar plates using a cork borer. Then, 100 µL of overnight culture of each indicator pathogen (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 64124), diluted to a turbidity corresponding to 0.5 McFarland, was spread on the agar plates, followed by the addition of 100 µL of each supernatant into the wells. The plates were then incubated at 37°C for 24 h, and the diameters (mm) of inhibition zones were subsequently measured. The test pathogens were sourced from the Laboratory of Molecular Biology and Biotechnology of the Pan African University Institute for Basic Sciences, Technology, and Innovation (Nairobi, Kenya).

### Antibiotic susceptibility test

The antibiotic susceptibility of the LAB isolates was evaluated using the disc diffusion method following the protocol outilined by Barzegar et al. (2021), with slight modifications. Briefly, overnight cultures of LAB isolates were diluted in PBS to a concentration equivalent to 0.5 McFarland (107-108 CFU/mL) and then spread on MRS agar plates. Ten antibiotic discs were placed on the agar plates, which were then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimetres (mm), and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2020). The antibiotic discs utilised in this study included erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), amoxicillin (30 µg), vancomycin (30 µg), amikacin (30 µg), ceftriaxone (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and ceftazidime (30 µg).

### Haemolytic activity

The haemolytic activity of the LAB isolates was assessed *in vitro* following the methodology outlined by Bazireh et al. (2020). Concisely, fresh bacterial cultures were streaked onto blood agar media supplemented with sheep blood (10% v/v) and the plates were then incubated for 24 h at 37°C. The appearance of colonies was examined, with beta haemolysis characterised by clear zones around the colonies and alpha haemolysis by greenish zones around the colonies. No change in the appearance of the agar indicated no hemolysis. *Listeria monocytogenes*, sourced from the Laboratory of Food Microbiology at JKUAT, served as a positive control.

### Molecular identification and phylogeny

Genomic DNA was extracted from selected isolates with probiotic potential using a Bacterial Genomic DNA Extraction Kit (Solarbio Co. Ltd., Beijing, China), following the manufacturer's instructions. PCR was performed to amplify the 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3') (Fan et al., 2022). The PCR reaction with a total volume of 25 µL, comprised 1 µL of both forward and reverse primers (10 µM), 12.5 µL of OneTaq Quick-Load 2X Master Mix (New England BioLab Inc., Ipswich, USA), 1.0 µL DNA template, and 9.5 µL Nuclease-free water. The PCR conditions were set as follows: initial denaturation at 94°C for 30 s, denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 68°C for 1 min, 30 cycles, and a final extension at 68°C for 5 min. The PCR products (1490 bp) were detected by 2% agarose gel electrophoresis. They were purified and sequenced by Macrogen Europe (Amsterdam, Netherlands). BioEdit 7.7.1 (Hall, 1999) was used for sequence analysis, and the sequence similarity comparison was performed using Basic Local Alignment Tool (BLAST) (Altschul et al., 1990) of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The sequences obtained from this study were aligned together with their closely related sequences in the NCBI database using Multiple Sequence Comparison by Log-Expectation (MUSCLE) software (Edgar, 2004). The phylogenetic tree was constructed using MEGA 11 (Tamura et al., 2021). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) at 1000 bootstrap replications.

### Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA). Duncan's post hoc test was performed to compare multiple means. Statistical analyses were conducted with SPSS 29.0.1.0 (IBMSPSS Statistics, Chicago, USA), and statistical significance was established at $p < 0.05$. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD) with superscript (a, b, c, d, and e).

### Ethical approval

This study was reviewed and approved by the JKUAT Institutional Scientific and Ethical Review Committee and granted the approval number JKU/ISERC/02316/1043.

## RESULTS

### Isolation and survival of LAB strains in gastrointestinal conditions

Five isolates (R11, R21, R52, R71 and R81) were cocci-shaped, Gram-positive,and catalase-negative. These strains underwent exposure to simulated gastric fluid (pH 2.5) and intestinal fluid (pH 8.0) to assess their survivability in gastrointestinal conditions. Remarkably, all five isolates were resilient under the simulated conditions

**Table 1.** Survival rate of LAB strains in simulated gastrointestinal conditions.

| Strain | Morphology | Gram | Catalase | log CFU/mL | | Survival rate in GIT (%) |
|---|---|---|---|---|---|---|
| | | | | 0 h* | 6 h** | |
| R11 | cocci | + | - | 8.21 ± 0.08$^{ab}$ | 7.17 ± 0.05$^{a}$ | 87.41 |
| R21 | cocci | + | - | 8.22 ± 0.13$^{ab}$ | 6.99 ± 0.06$^{b}$ | 85.04 |
| R52 | cocci | + | - | 8.38 ± 0.08$^{a}$ | 6.89 ± 0.05$^{b}$ | 82.25 |
| R71 | cocci | + | - | 8.30 ± 0.03$^{ab}$ | 6.35 ± 0.10$^{d}$ | 76.51 |
| R81 | cocci | + | - | 8.14 ± 0.05$^{b}$ | 6.57 ± 0.03$^{c}$ | 80.68 |

*CFU counts before the *in vitro* gastrointestinal survival test. **CFU counts after6 hours of simulated gastrointestinal conditions. Data are expressed as mean ± SD. Mean with different superscripts in a column are significantly different (p<0.05).

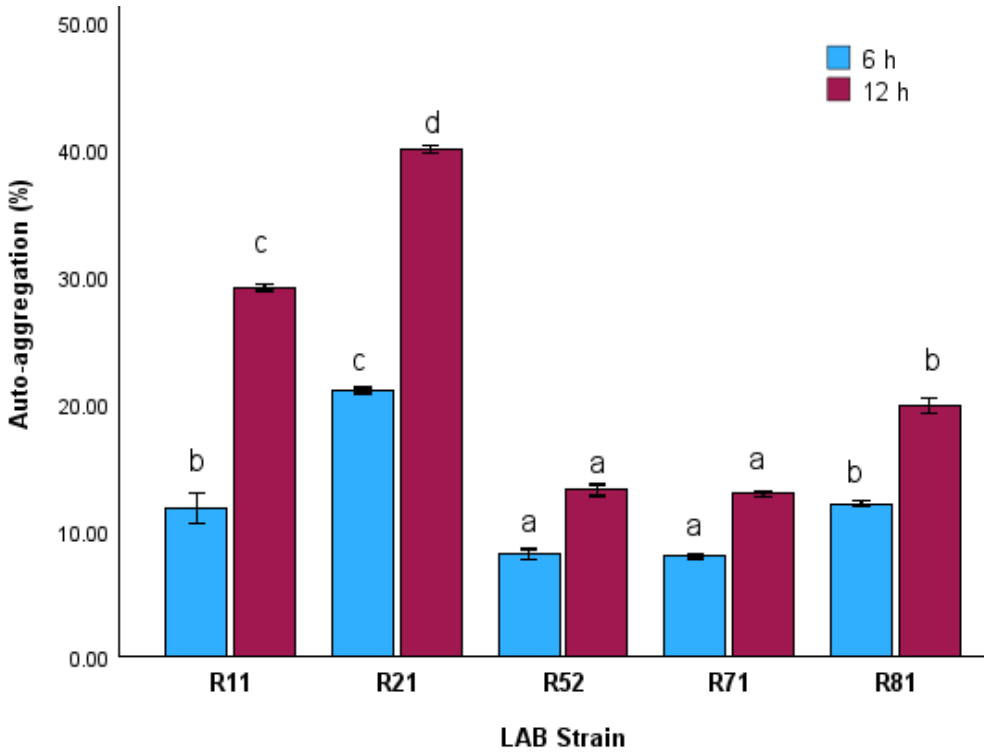


**Figure 1.** Auto-aggregation of the LAB strains after 6 and 12 h of incubation. Means with different superscript letters are significantly different (p<0.05), letter "a"being the lowest mean.

for 6 h, enduring the challenges posed by the low pH, digestive enzymes, and bile salt concentration (Table 1). Among the strains, R11 and R71 had the highest (87.41%) and lowest (76.51%) survival rates, respectively.

## Auto-aggregation

The auto-aggregation ability of the lactic acid bacteria strains was assessed at 6 and 12 h after incubation. All five LAB strains were able to auto-aggregate at different rates after 6 and 12 h of incubation, and the auto-aggregation rates increased with the incubation time. Strain R21 exhibited the highest auto-aggregation rate (21%) after 6 h of incubation, which increased to 40% after 12 h. The lowest auto-aggregation rates were observed in R52 and R71 (Figure 1).

## Antimicrobial activity

The antimicrobial activity of the cell-free supernatant from the lactic acid bacteria strains was assessed against four indicator pathogens, and the results were presented as the diameters of zones of inhibition. All strains exhibited inhibitory activity against all indicator pathogens to varying

**Table 2.** Antimicrobial activity of Lactic acid bacteria isolates.

| Strain | Zone of inhibition (mm) against indicator pathogens | | | |
|---|---|---|---|---|
| | ***E. coli* ATCC 25922** | ***S. aureus* ATCC 43300** | ***P. aeruginosa* ATCC 27853** | ***C. albicans* ATCC 64124** |
| R11 | 20.33±0.58$^{a}$ | 16.33± 0.58$^{b}$ | 11.00±0.00$^{c}$ | 9.33 ± 0.58$^{c}$ |
| R21 | 16.67±0.58$^{b}$ | 14.33 ±0.58$^{c}$ | 12.00±0.00$^{b}$ | 11.00 ± 0.58$^{b}$ |
| R52 | 18.00±0.00$^{b}$ | 23.33± 0.58$^{a}$ | 9.33± 0.58$^{d}$ | 10.33 ± 0.58$^{bc}$ |
| R71 | 20.33±0.58$^{a}$ | 15.33± 0.58$^{bc}$ | 13.00±0.00$^{a}$ | 11.33 ± 0.58$^{b}$ |
| R81 | 14.67±0.58$^{c}$ | 15.33± 0.58$^{bc}$ | 11.33±0.58$^{bc}$ | 13.67 ± 0.58$^{a}$ |

Low activity:>7.8 mm; moderate activity: ≥ 13 mm; strong activity: ≥ 20 mm. Data are expressed as mean value ± SD. Means with different superscripts in a column are significantly different ($p<0.05$).

**Table 3.** Antibiotic sensitivity of Lactic acid bacteria isolates.

| Strain | CIP | AK | CAZ | AMP | E | GEN | VAN | CRO | AMX | TE |
|---|---|---|---|---|---|---|---|---|---|---|
| R11 | S | **R** | I | S | I | **R** | S | S | S | S |
| R21 | S | **R** | **R** | S | S | **R** | S | S | S | S |
| R52 | S | **R** | S | S | S | I | S | S | S | **R** |
| R71 | S | **R** | S | S | S | S | S | S | S | **R** |
| R81 | S | **R** | I | S | I | **R** | S | S | S | S |

CIP= Ciprofloxacin 30 µg, AK= Amikacin 30 µg, CAZ= Ceftazidime 30 µg, AMP= Ampicillin 10 µg, E= Erythromycin 15 µg, GEN= Gentamicin 10 µg, VAN= Vancomycin 30 µg, CRO= Ceftriaxone 30 µg, AMX= Amoxycillin 30 µg, TE= Tetracycline 30 µg, S= Sensitive, R= Resistant, and I= Intermediate sensitivity.

extents (Table 2). Notably, the neutral supernatants of R11 and R71 showed strong inhibitory activity against *E.* coli, while R2 showed strong inhibition against *S. aureus*. Four strains (80%) showed low inhibitory activity against *C. albicans*.

### Antibiotic susceptibility

The susceptibility of the lactic acid bacteria strains to ten different antibiotics was assessed using the disc diffusion method. All five strains were sensitive to Ciprofloxacin, Ampicillin, Vancomycin, Ceftriaxone, and Amoxicillin (Table 3). However, all strains were resistant to Amikacin, and three strains (R11, R21, and R81) were also resistant to Gentamicin. Additionally, two strains (R52 and R71) were found resistant to Tetracycline.

### Haemolytic activity

All five lactic acid bacterial isolates did not exhibit haemolytic activity, in contrast to the positive control, which showed a haemolytic reaction (Figure 2).

### Molecular identification and phylogenetic analysis

BLAST analysis was performed for sequence similarity search. The isolates R11 and R52 showed 99% similarity with *Enterococcus faecalis* 12YGD (OQ123535) and *Lactococcus garvieae* J (MT640284), respectively. Isolates R21, R71, and R81 showed 100% similarity with *Enterococcus hirae* 1104 (MT626063), *L. garvieae* F (MT640282), and *E. faecalis* 2623 (MT611645), respectively. The five strains were part of three major clades, including *E. faecalis*, *E. hirae*, and *L.garvieae*, as indicated by the phylogenetic analysis (Figure 3). The sequences generated were submitted to GenBank and assigned accession numbers are indicated in Table 4.

## DISCUSSION

Five types of lactic acid bacteria were found to be possible probiotic strains in this study. They are *E. faecalis* R11, *E. faecalis* R81, *E. hirae* R21, *L. garvieae* R52, and *L. garvieae* R71. Previous research has suggested that lactic acid bacteria such as *Enterococcu*s strains isolated from human faeces and saliva (Bazireh et al., 2020), *L. garvieae* from healthy piglets (Zhang et al., 2016), *Enterococcus hirae* from healthy Chinese infants (Wei et al., 2020), and *E. faecalis* from Iranian fermented dairy product, Kashk (Saboori et al., 2022) are promising probiotic candidates. *Enterococcus* strains, naturally present in the gut of both humans and animals, can survive, compete, and adhere to host cells in the gastrointestinal tract (GIT). This is crucial for their effective utilisation as probiotics (Hanchi et al., 2018).

High survival rate during gastrointestinal transit is a

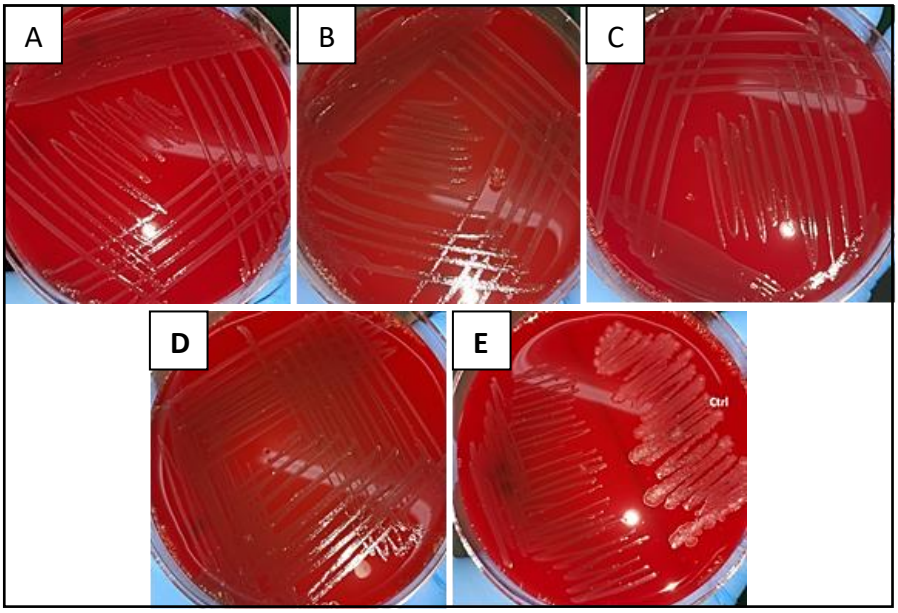


**Figure 2.** Haemolytic activity of LAB isolates. A: strain R11; B: strain R21; C: strain R52; D: strain R71; E: strain R81; and Ctrl= positive control (*Listeria monocytogenes*).

crucial feature as it indicates the potential of a probiotic bacterial strain. This means that the probiotic strains should be able to withstand the acidity and bile concentrations in the colon to effectively provide its beneficial health effects to the consumer (Celiberto et al., 2018). Factors such as low pH levels can impede metabolism and reduce the growth and viability of LAB. Gastric cells release highly acidic gastric secretions, establishing stringent conditions for the viability of bacteria passing through the stomach (Saboori et al., 2022). In our study, all identified LAB strains exhibited high survival rates in simulated gastric and intestinal juices, with *E. faecalis* R11 showing the significantly highest survival rate (Table 1). Our experimental results demonstrated that *Lactococcus* and *Enterococcus* strains isolated from rat faeces could withstand extreme acidic pH (pH 2.5), basic pH (pH 8), and survived in high concentrations of bile salt (1%). These findings align with previous studies where *Enterococcus* strains (Baccouri et al., 2019) and *L. garvieae* strains (Patel et al., 2020) were viable in acidic pH and high bile salt concentrations. Our results suggest that these lactic acid bacteria isolates can survive in the human and animal gastrointestinal tract and likely withstand passage through the stomach and intestines.

Auto-aggregation is another essential criterion to consider when selecting potential probiotic candidates. The ability of microorganisms to aggregate is directly related to their ability to adhere to and colonise the gastrointestinal tract of the host (Byakika et al., 2019). In the present study, the auto-aggregation rate of all the strains increased with the incubation time. The highest auto-aggregation rates were observed in *E. hirae* R21 (40%), followed by *E. faecalis* R11 (29.11%), and *E. faecalis* R81 (19.81%). The *L. garvieae* strains showed the lowest autoaggregation rates. The *Enterococcus* and *L. garvieae* strains isolated in this study exhibited higher auto-aggregation rates after a 12-h incubation period than *Lactobacilli* strains isolated from rat faeces by Jena et al. (2013). These strains have the ability to impact the immune system, outcompete pathogens for binding to the intestinal epithelial receptor cells, and reduce the presence of harmful intestinal microorganisms (Nascimento et al., 2019).

Antimicrobial activity is one of the key features to consider when evaluating the probiotic potential of microorganisms (Byakika et al., 2019). The production of organic acids, hydrogen peroxide, phenols, diacetyl, proteins, and probiotic development itself may contribute to probiotics' antimicrobial action. These metabolites, along with a competitive exclusion mechanism, help probiotics eliminate and prevent pathogenic microbes from colonising the body by competing with them for adhesion and resources (Aditya et al., 2020). In this study, the neutral cell-free supernatants of the strains exhibited varying degrees of inhibitory activity against all the indicator pathogens. Our results align with those of Jena et al. (2013) in which all the lactic acid isolated from rat faeces inhibited the growth of all the pathogens to different extents. *E. hirae* R52 showed the strongest inhibitory activity against methicillin-resistant *S. aureus* (MRSA) ATCC43300. These findings emphasize the importance of the isolated *Enterococcus* and *Lactococcus* strains in this study, as they have a broad spectrum of

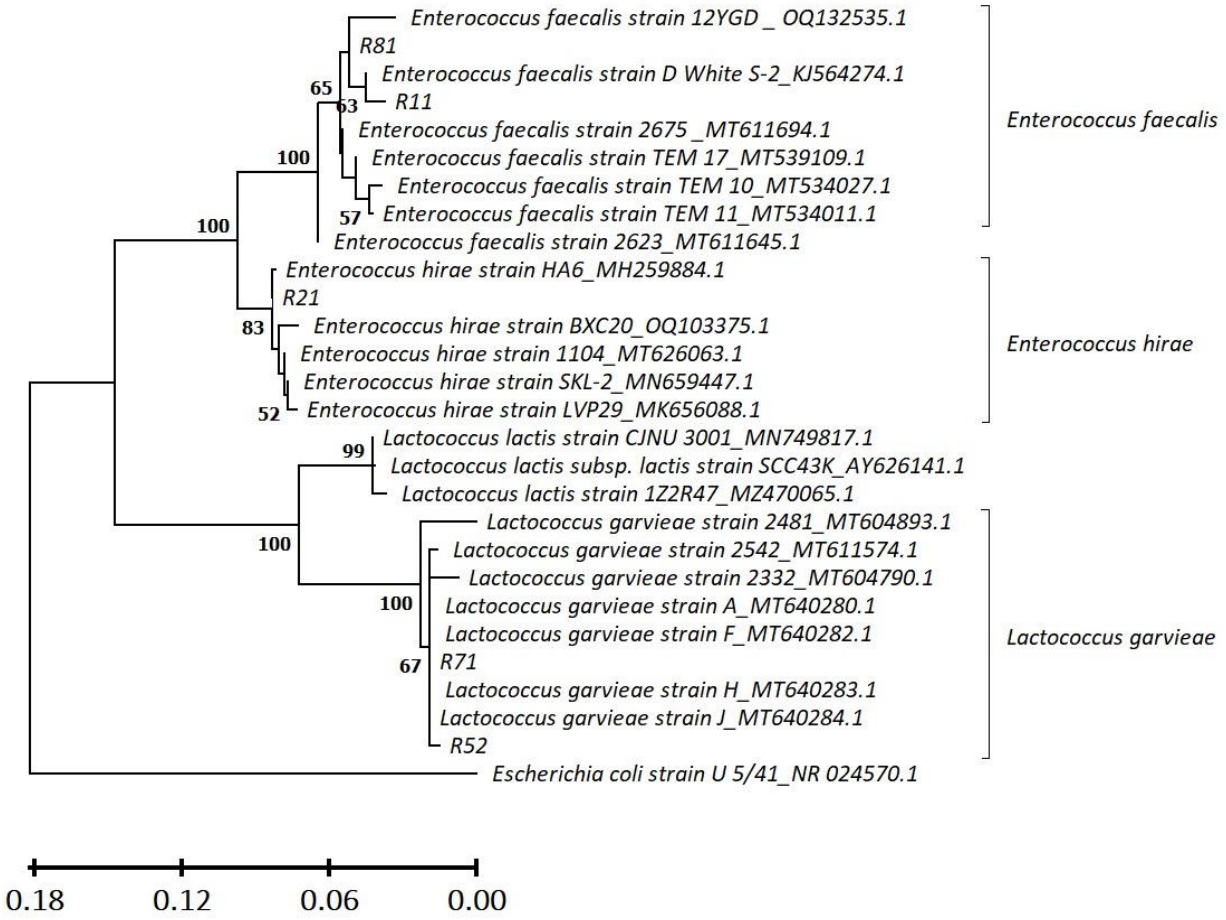


**Figure 3.** Maximum-Likelihood phylogenetic trees showing the taxa related to the isolated *Enterococcus* and *Lactococcus* strains. *Escherichia coli* U 5/41 was used as an outgroup.

**Table 4.** Molecular identification of the isolated LAB strains.

| Strain | Species | NCBA Accession No. |
|---|---|---|
| R11 | *Enterococcus faecalis* | OR921269 |
| R21 | *Enterococcus hirae* | OR921270 |
| R52 | *Lactococcus garvieae* | OR921272 |
| R71 | *Lactococcus garvieae* | OR921273 |
| R81 | *Enterococcus faecalis* | OR921274 |

antimicrobial activity, particularly against MRSA and the fungus *C. albicans*.

Evaluating the antibiotic susceptibility is crucial to ensuring the safety of a potential probiotic candidate. The potential transfer of antibiotic resistance from probiotic strains to pathogenic members of the microbiota, either directly or through intermediary microorganisms, is a worrisome issue as it may lead to the development of antibiotic-resistant pathogens that are difficult to treat (Roe et al., 2022). Based on the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2020), all strains in this study were susceptible to ciprofloxacin, ampicillin, vancomycin, ceftriaxone, and amoxicillin. In this study, *Enterococcus* strains were resistant to gentamicin and sensitive to vancomycin, which is consistent with the findings of Baccouri et al. (2019). The sensitivity to vancomycin was attributed to the absence of vancomycin resistance genes in the genomic analysis of *Enterococcus* strains. Resistance to gentamicin was previously described as intrinsic (Baccouri et al., 2019) and therefore cannot be transmitted to other bacteria.

Haemolysis is one of the two main virulence factors of pathogenic bacteria (Halder et al., 2017). Strains with haemolytic activity have the ability to cause anaemia, bacteraemia, and oedema, posing a significant risk to the consumer's health (Qin et al., 2023). Therefore, it is crucial to ensure that a microorganism intended for use as a probiotic does not exhibit haemolytic activity. In this study, none of strains exhibited haemolytic activity. The absence of haemolytic activity is another necessary safety criterion when selecting a probiotic strain since it indicates that the bacteria are not harmful, and the absence of haemolysin prevents the emergence of opportunistic virulence among the strains (Casarotti et al., 2017).

## Conclusion

In this study, five strains of lactic acid bacteria with potential probiotic properties were successfully isolated from rat faecal samples, identified, and characterised. These strains, *E. faecalis* R11, *E. hirae* R21, *L. garvieae* R52, *L. garvieae* R71, and *E. faecalis* R81, had good probiotic traits such as being able to survive in simulated gastrointestinal fluids, clumping together on their own, and inhibiting the growth of indicator pathogens. The strains were also susceptible to a range of antibiotics and non-haemolytic, making them safe for use not only in studies involving rat models but also in industries and human as well as animal health. However, further *in vivo* studies are required to assess their safety and putative health benefits.

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## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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