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Impact of Environmental Factors on *Clostridium perfringens* Sporulation Isolated from Poultry Droppings-Contaminated Soils in the of Poultry Farms

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ABSTRACT

The persistence and pathogenicity of *Clostridium perfringens*, a spore-forming anaerobic bacterium, in poultry farm environments pose significant risks to animal health and food safety. This study aimed to investigate the influence of environmental factors—such as humidity, pH, temperature, and oxygen availability—on the growth and sporulation of *C. perfringens* isolated from poultry droppings-contaminated soils in the Malwa region of Madhya Pradesh, India. A total of 63 soil samples were collected from poultry farms across Ratlam, Mandsaur, Ujjain, and Indore. Five isolates (7.94%) tested positive for *C. perfringens* through biochemical, morphological, and phenotypic characterization. Controlled laboratory experiments revealed that optimal growth occurred at 35°C and pH 7.0 under strictly anaerobic conditions, with significant enhancement of sporulation at high humidity levels (90% RH). Antibiotic susceptibility profiling showed highest sensitivity to Ciprofloxacin, Rifampin, and Chloramphenicol, while resistance was observed against Amoxicillin, Gentamicin, and Vancomycin. These findings highlight the environmental resilience of *C. perfringens* and emphasize the importance of environmental management and targeted antibiotic use in poultry farming.

1. INTRODUCTION

The poultry industry is a vital contributor to food security and economic stability. It also contributes significantly to the rural economy by creating productive work opportunities and boosting family income, especially for women, small and marginal farmers, and landless labourers (Hussein et al., 2021). Therefore, it is important to fulfil demand by importing improved and healthy breeds of poultry. But right now, the most major problem is the occurrence of numerous bacterial, viral, and parasitic internal and external diseases in which many different types of microorganisms can be spread by poultry droppings (Wachsmuth, 1986). Feed material is inoculating in the environment by transferring soil to standing crops through wind, rain, mechanical agitations, and insects which leads to significant environmental concerns, especially regarding waste management and soil contamination (Maciorowski et al., n.d.). Poultry droppings, rich in organic matter and nutrients, are often used as soil amendments or inadvertently deposited into agriculture lands (Hutchison et al., n.d.). Soil serves as a critical reservoir for microbial communities, including both beneficial and pathogenic (Samaddar et al., 2021). While poultry manure is valued for its fertilization properties, certain bacteria contribute to desiccated and nutrient-poor soil conditions (Kobierski et al., n.d.). Gastrointestinal pathogens can also be introduced into the food chain by poultry waste defecating in the farm environment or by fertilization of crops with manures (de Mesquita Souza Saraiva et al., 2022). Some anaerobic spore-bearing bacteria, such as *Clostridium* species, can exist as vegetative cells or spores, making them equally adept at surviving in soil and gastrointestinal systems (Araujo et al., n.d.). *Clostridium. perfringens*

and *Clostridium. botulinum* are two anaerobic Clostridium species that are of significant concern in feed (Bhunia et al., 2018).

The presence of *Clostridium perfringens* in poultry droppings contaminated soils poses different challenges to soil health, microbial diversity and agricultural productivity (Van Immerseel et al., 2004). *Clostridium perfringens*, which causes necrotic enteritis, a disease detrimental to poultry health and farm economics (Thompson et al., 2012). It can persist in soil and potentially contaminated crops. *Clostridium perfringens* is a gram-positive, spore-forming, anaerobic bacterium commonly found in the gut of poultry in an inactive state (EI-Jakee et al., 2013). But in certain conditions, it releases excessive type A and NetB toxins and, to a lesser extent, type C toxins in the small intestine, causing mucosal damage to the small intestine and playing a role in the pathogenesis of the infection of necrotic enteritis (Keyburn et al., 2008). Under favorable environmental condition, this bacterium can thrive in soil, forming resilient spores that can survive in extreme condition (Araujo et al., n.d.). Poultry farm soils, often enriched with organic waste, provide an ideal habitat for *C. perfringens* due to their moisture content, organic matter availability, and anaerobic microenvironments. The persistence of *C. perfringens* in contaminated soils raises concerns regarding its potential transmission through the soil-crop-food chain, impacting agricultural productivity and food safety (Hutchison et al., n.d.).

Environmental factors play an important role in the growth, survival, and even the pathogenicity of *C. perfringens* pH, temperature, humidity, and even incubation environments greatly affect the viability of bacteria and the overall production of toxins (Mehdizadeh Gohari et al., 2021). The soils of poultry farms that are contaminated with droppings provide harsh conditions that *C. perfringens* requires to thrive and even act as a reservoir for the bacterium. *C. perfringens* can live within slightly acidic to neutral pH levels, mesophilic temperatures, and moist environments(Medical & 1981, n.d.). These conditions are similar to the poultry gastrointestinal tract, allowing the transmission and colonization of the bacteria. Understanding the environmental factors that affect the establishment and survival of *C. perfringens* is essential for developing effective control strategies. Differences in temperature and humidity, for instance, can influence the formation of spores, which contribute to the bacterium's resilience and ability to cause recurrent infections. *C. perfringens* was also shown to have a direct relation with the levels of available oxygen during incubation that resulted in metabolism and growth; this furthered the importance of anaerobic management in laboratories and farms (Tsiouris et al., 2018).

Another major issue is the use of antibiotics in poultry farming regularly to reduce the chances of bacterial infection and to stimulate growth (Rood et al., 1978). The excessive and indiscriminate use of antibiotics has led to the emergence of antibiotic-resistant strains of *C. perfringens*, complicating disease control and treatment strategies. Resistance to commonly used antibiotics such as cephalosporins and ciprofloxacin has been reported, limiting the effectiveness of traditional therapeutic approaches (J. Chen et al., 2024). Understanding the susceptibility patterns of *C. perfringens* to various antibiotics is crucial for managing bacterial infections in poultry farms and preventing the spread of resistant strains. (Asha et al., 2006)

This study aims to investigate the impact of environmental factors, such as pH, temperature, humidity, and incubation conditions, on the growth and survival of *C. perfringens* isolates from contaminated soils in poultry farms across the Malwa region of India. Further, the study investigates the susceptibility of these isolates to commonly used antibiotics to assess resistance patterns. By understanding the interplay between environmental factors and antibiotic susceptibility, this research seeks to provide insights into the persistence and resistance mechanisms of *C. perfringens*, contributing to more effective control strategies and sustainable poultry farming practices, sustainable poultry waste management, soil health conversation, and food safety strategies.

2. MATERIALS AND METHODS

Sample Collection from poultry droppings-contaminated soils

In a descriptive study, a total of 63 samples contaminated with poultry droppings were collected from identified poultry farms located in various areas of Indore, Mandsaur, and Ratlam in the Malwa region of Madhya Pradesh. These districts were selected due to their extensive poultry production and reported instances of clinical symptoms in birds. A total of 21 poultry farms were selected for sample collection, distributed as follows: 4 in Ratlam, 6 in Mandsaur, 3 in Ujjain, and 8 in Indore, as mentioned in **Table 1.**

Table 1: Distribution of Poultry Farms Selected for Sample Collection in Different Districts of the Malwa Region, Madhya Pradesh

Sample Type	Sample Code	No of Sample	Location	No. of Poultry Farms	Season
Poultry droppings-contaminated soils (63 Samples)	PDCS 1 to 12	12	Ratlam	4	Rainy Season
	PDCS 13 to 30	18	Mandsaur	6	

	PDCS 31 to 39	09	Ujjain	3	
	PDCS 40 to 63	24	Indore	8	

Three samples were taken from each farm. Before collecting the samples, all equipment including gloves, spatulas, and airtight containers, was sterilised. Disinfectants like 70% ethanol were used to clean the tools between collections to prevent cross-contamination. Soil samples were taken from the top 5–10 cm layer of the ground, as this layer is most likely to be contaminated by poultry droppings. Approximately 50–100 grams of soil were collected from different points within each farm to create a composite sample, ensuring it accurately represented the area. The samples were then placed in sterile, airtight containers and transported under cool conditions (around 4°C) using ice packs to maintain the viability of the bacteria. Care was taken to avoid freezing the samples, as freezing could damage bacterial cells. All samples were processed within 24–48 hours of collection. If processing was delayed, the samples were stored at 4°C to preserve microbial integrity. Detailed documentation was maintained for each sample, including the farm's location, environmental conditions (such as temperature and humidity), soil type, and poultry management practices, to help establish correlations during the analysis. Control samples from soil not exposed to poultry but under similar environmental conditions were also collected to serve as a baseline for comparison.

Isolation and Identification of *C. perfringens* from droppings-contaminated soils.

For the isolation and identification of *C. perfringens*, the droppings-contaminated soil samples were first enriched in Robertson Cooked Meat (RCM). A 1 g aliquot of each soil sample was suspended in 9 mL of sterile RCM broth and incubated anaerobically at 37°C for 24–48 hours (Miah et al., 2011). As a result, the growth of *Clostridium perfringens* is observed with vigorous gas bubbles, turbidity, and a sour smell with redness protein because *Clostridium perfringens* is saccharolytic anaerobes that cause rapid production of acid, gas and no digestion of meat. Following enrichment, a loopful of the culture was streaked onto Sheep Blood Agar (SBA) plates prepared with 5% defibrinated sheep blood (Rahaman et al., n.d.). These plates were incubated anaerobically at 37°C for 24–48 hours using an anaerobic jar with gas-generating sachets to maintain a low-oxygen environment. Colonies characteristic of *C. perfringens*—smooth, round, and displaying a double zone of hemolysis—were identified visually. For identification of *Clostridium perfringens* involved morphological, biochemical, and phenotypical tests. For morphological identification, motility testing was performed using semi-solid motility medium (Tizhe et al., 2015). Non-diffused growth along the stab line indicated non-motility. Gram staining (Jensen's modification) showed Gram-positive bacteria appearing blue. Spore staining using the Schaeffer-Fulton method revealed green endospores within pink vegetative cells. Hemolytic activity was assessed on sheep blood agar, identifying alpha and beta hemolysis patterns (Microbiology & 1978, 1978).

Biochemical tests included gelatin liquefaction (positive), litmus milk fermentation (stormy fermentation), and nitrate reduction (red color indicated nitrite formation). The urease, oxidase, catalase, and indole tests were negative. Hydrogen sulfide (H_2S) production was indicated by black precipitate in TSI agar, while citrate utilization showed a blue color on Simmon's citrate agar. The TSI test confirmed sugar fermentation, gas, and H_2S production (Stark et al., 1972). Phenotypical tests included DNase enzyme activity (clear zones on DNase agar), lecithinase activity (opaque halos on egg yolk agar), and hemolysin production (alpha and beta hemolysis on blood agar) (Rahman et al., n.d.). After detecting positive sample for *Clostridium perfringens* strain, they can be preserved on sheep blood agar plate at -80°C in 20% glycerol.

Effect of Environmental Factors on the Growth of *Clostridium Perfringens*-Positive Samples Isolated from Poultry Dropping-Contaminated Soils.

The Effect of environmental factors on *Clostridium perfringens* positive samples involves understanding how variables such as humidity, incubation pH and temperature, conditions influence its growth, sporulation, and toxin production in which all tests are performed in triplicates.

1. **Humidity:** - Humidity plays a crucial role in bacterial growth by influencing the moisture content of the environment, as desiccation can significantly inhibit the proliferation of *Clostridium perfringens* (preservation & 2012, 2012). The materials used in this study included a *Clostridium perfringens* strain, which was revived from a -80°C glycerol stock and cultured on Sheep Blood Agar (SBA) plates (S. Chen et al., 2017). Thioglycollate Broth (TGB) was used for initial bacterial enrichment, while *C. perfringens* Sporulation Broth (CPSB) served as the primary growth medium for humidity experiments (Alhabeeb, 2021). To create controlled humidity environments, a desiccator with saturated salt solutions was employed, using potassium acetate (KAc) for 23% relative humidity (RH), magnesium nitrate ($Mg(NO_3)_2$) for 53% RH, sodium chloride (NaCl) for 75% RH, and potassium chloride (KCl) for 85% RH (Science & 2022, 2022). Sterile anaerobic tubes and flasks were used to maintain culture sterility, and an anaerobic chamber or sealed incubation system was utilized to ensure oxygen-free conditions during bacterial growth. A hygrometer was placed inside the desiccator to continuously monitor and regulate humidity levels throughout the experiment.

Growth was measured using a spectrophotometer at OD600, while sporulation was assessed using Schaeffer-Fulton spore staining and heat shock treatment followed by plating on Tryptose Sulfite Cycloserine (TSC) Agar (Devi et al., n.d.). These materials ensured precise control over environmental factors to evaluate the effect of humidity on *C. perfringens* growth

Culture Preperation :-
A previously preserved *C. perfringens* strain stored at -80°C in 20% glycerol was revived by streaking onto SBA plates and incubating anaerobically at 37°C for 16–24 hours. A single colony was then transferred into Thioglycollate Broth (TGB) and incubated anaerobically at 37°C for 12–18 hours until visible turbidity was observed. Subsequently, 1 mL of the actively growing culture was inoculated into 9 mL of CPSB (1:10 dilution) for humidity experiments.

Humidity Control Using a Desiccator with Saturated Salt Solutions: - To create different relative humidity (RH) conditions, a sealed desiccator containing saturated salt solutions was used. Specific salts were chosen to maintain stable humidity levels: potassium acetate (23% RH), magnesium nitrate (53% RH), sodium chloride (75% RH), and potassium chloride (85% RH). The inoculated CPSB tubes were placed inside the desiccator along with the respective salt solutions and allowed to equilibrate before incubation. A hygrometer was placed inside the desiccator to monitor and maintain the required humidity levels throughout the experiment.

Growth and Sporulation Assessment: -
The cultures were incubated anaerobically at 37°C for up to 48 hours under different humidity conditions (50%, 70%, and 90% RH). Growth was monitored by measuring optical density (OD600) at regular intervals to assess bacterial proliferation under varying humidity levels. Sporulation was evaluated using Schaeffer-Fulton staining to visualize spore formation and heat shock treatment (80°C for 10 minutes), followed by plating on TSC Agar for spore enumeration

Data Analysis:-
The OD600 values were plotted over time to generate growth curves of *C. perfringens* at different humidity levels. The effect of humidity on sporulation efficiency was analyzed based on the number of heat-resistant spores formed under each condition. Statistical analysis was performed to determine significant differences in growth and sporulation among varying humidity levels.

This experiment provides insights into how environmental humidity influences *C. perfringens* growth and sporulation, which is crucial for understanding its adaptability and potential implications in poultry farming and food safety.

2. Incubation: - The incubation conditions for *Clostridium perfringens* were evaluated to compare its growth under strictly anaerobic and microaerophilic environments, providing insights into its oxygen tolerance and ecological adaptability (Clifford et al., 1974). As an obligate anaerobe, *C. perfringens* thrives in oxygen-free environments but can tolerate limited oxygen exposure, with reduced growth rates at redox potentials up to +200 mV
A previously preserved *C. perfringens* strain stored at -80°C in 20% glycerol was revived by streaking onto SBA plates and incubating into different incubation condition.
Duplicate sets of streaked sheep blood agar plates were incubated under two distinct environmental conditions. One set was incubated **under strictly anaerobic conditions** in anaerobic jars equipped with gas-generating sachets to maintain an oxygen-free atmosphere (Stevens et al., 1987). The other set was incubated under **microaerophilic conditions** using a controlled environment with reduced oxygen levels to simulate low-oxygen conditions. Both sets were incubated at 37°C for 24–48 hours.

Growth Assessment: After incubation, bacterial growth was assessed by measuring colony-forming units (CFUs) on agar plates. Comparative analysis of growth between anaerobic and microaerophilic conditions provided quantitative data on the oxygen tolerance of *C. perfringens*. As expected, optimal growth was observed under anaerobic conditions, confirming the bacterium's obligate anaerobic nature. Negligible or significantly reduced growth was noted under microaerophilic conditions, consistent with the inhibitory effect of oxygen exposure. These findings highlight the critical role of strict anaerobic environments in supporting the growth and metabolic activity of *C. perfringens*. The study provides valuable insights into the bacterium's ecological preferences and adaptability, which are essential for devising strategies to control its spread and mitigate its impact on food safety and disease management.

3. Temperature & Ph: - This study utilized a *Clostridium perfringens* strain, which was revived from a -80°C glycerol stock stored in 20% glycerol. The culture was streaked onto Sheep Blood Agar (SBA) plates and incubated anaerobically at 37°C for 16–24 hours. Thioglycollate Broth (TGB) was used for bacterial enrichment, while *C. perfringens* Sporulation Broth (CPSB) served as the primary medium for growth experiments (Nasir et al., 2019). To assess the effect of temperature, an incubator with adjustable temperature settings was used, while pH adjustments were made using 1N HCl and 1N NaOH. Sterile anaerobic tubes and flasks were used to maintain culture sterility, and an anaerobic chamber

ensured oxygen-free conditions. A spectrophotometer was used to measure optical density at 600 nm (OD600), and pH was monitored using a calibrated pH meter. Growth curves were analyzed to determine the optimal environmental conditions for *C. perfringens* proliferation.

Culture Preparation
The *C. perfringens* strain was streaked from a -80°C glycerol stock onto SBA plates and incubated at 37°C for 16–24 hours under anaerobic conditions. A single colony was then transferred into 10 mL of Thioglycollate Broth (TGB) and incubated anaerobically at 37°C for 12–18 hours until visible turbidity was observed. Subsequently, 1 mL of the culture was inoculated into 9 mL of CPSB for temperature and pH experiments.

Temperature Experiment Setup: -
To evaluate the effect of temperature on *C. perfringens* growth, inoculated CPSB cultures were incubated under anaerobic conditions at six different temperatures: 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C. Growth was monitored at regular intervals over 48 hours by measuring OD600 using a spectrophotometer. Growth curves were generated to determine the temperature range supporting maximum bacterial proliferation.

pH Experiment Setup:-
For pH analysis, CPSB media was adjusted to different pH levels (3.0, 5.0, 7.0, 9.0, and 11.0) using 1N HCl or 1N NaOH before sterilization. The pH-adjusted media was inoculated with *C. perfringens* and incubated anaerobically at 37°C for 48 hours. Growth was assessed at different time points by measuring OD600. The final pH of each culture was measured post-incubation to analyze any changes due to bacterial metabolism.

Growth and Data Analysis
Growth curves were generated for both temperature and pH conditions. The OD600 values were recorded at regular intervals to determine bacterial proliferation trends. Statistical analysis was conducted to compare growth differences across temperature and pH conditions, providing insight into the optimal environmental factors for *C. perfringens* growth.

4. Antimicrobial Susceptibility Testing of *Clostridium perfringens* Isolated from Poultry Droppings-Contaminated Soils: -
The study was conducted using a *Clostridium perfringens* strain revived from a -80°C glycerol stock and cultured on Sheep Blood Agar (SBA). Thioglycollate Broth (TGB) was used for bacterial enrichment, and Mueller-Hinton Agar (MHA) was used for antibiotic susceptibility testing. The antibiotics tested included Clindamycin, Rifampin, Tetracycline, Amoxicillin, Erythromycin, Chloramphenicol, Ciprofloxacin, Gentamicin, and Vancomycin. Antibiotic discs were obtained commercially, and the disc diffusion method (Kirby-Bauer method) was used to assess bacterial susceptibility. A sterile cotton swab, forceps, and an incubator set at 37°C under anaerobic conditions were also used. Zone of inhibition measurements were recorded using a digital caliper

Culture Preparation
The *C. perfringens* strain was streaked onto SBA and incubated anaerobically at 37°C for 16–24 hours. A single colony was transferred into 10 mL of TGB and incubated at 37°C for 12–18 hours until visible turbidity was observed. The bacterial suspension was adjusted to 0.5 McFarland standard (~1.5×10^8 CFU/mL) using sterile saline.

Antibiotic Susceptibility Testing (Disc Diffusion Method)
Mueller-Hinton Agar (MHA) plates were inoculated with the standardized bacterial suspension using a sterile cotton swab. Antibiotic discs were placed onto the inoculated plates using sterile forceps, ensuring even distribution. The plates were incubated anaerobically at 37°C for 24 hours. Following incubation, the zone of inhibition (diameter in mm) around each antibiotic disc was measured using a digital caliper(J. Chen et al., 2024).

Data Analysis
The measured inhibition zones were compared among different antibiotics to determine the effectiveness of each against *C. perfringens*. The results were classified as resistant, intermediate, or susceptible based on standard Clinical and Laboratory Standards Institute (CLSI) guidelines

3. RESULTS

Isolation and Identification of *C. perfringens* from droppings-contaminated soils

Sample enrichment was performed in Reinforced Clostridial Medium (RCM) under anaerobic conditions, followed by culturing on selective media. Observations included turbidity of the medium, gas bubble formation, a foul sour odor, and reddening of the protein content, reflecting saccharolytic activity—a metabolic trait associated with *Clostridium perfringens*—as shown in **Figure 1**. Characteristic growth of *Clostridium perfringens* was observed, indicated by smooth, rounded colonies exhibiting double-zone hemolysis on blood agar. The colonies appeared smooth, convex, moist, and

measured 2–4 mm in diameter, as shown in **Figure 2.** Positive isolates exhibited saccharolytic activity with rapid gas and acid production, confirming their identity. **Control soil samples from non-contaminated areas showed no growth of *C. perfringens*, reinforcing its association with poultry contamination.** Isolation and identification were carried out through a series of morphological, biochemical, and phenotypic tests. The results for representative samples (PDCS 12, PDCS 21, PDCS 45, PDCS 50, and PDCS 63) are summarized in **Tables 2.1 and 2.2.** Additionally,

Figure 3 presents a stacked graph showing the number of samples positive for different tests.

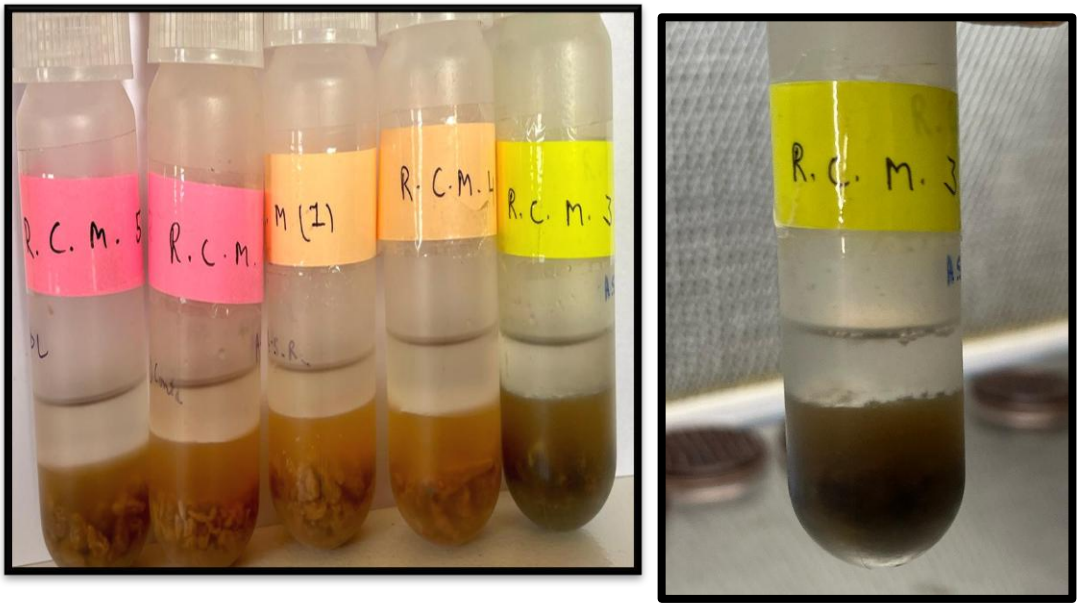

Figure 1: Characteristic gas bubble formation, turbidity and protein reddening indicating saccharolytic activity of *Clostridium perfringen*

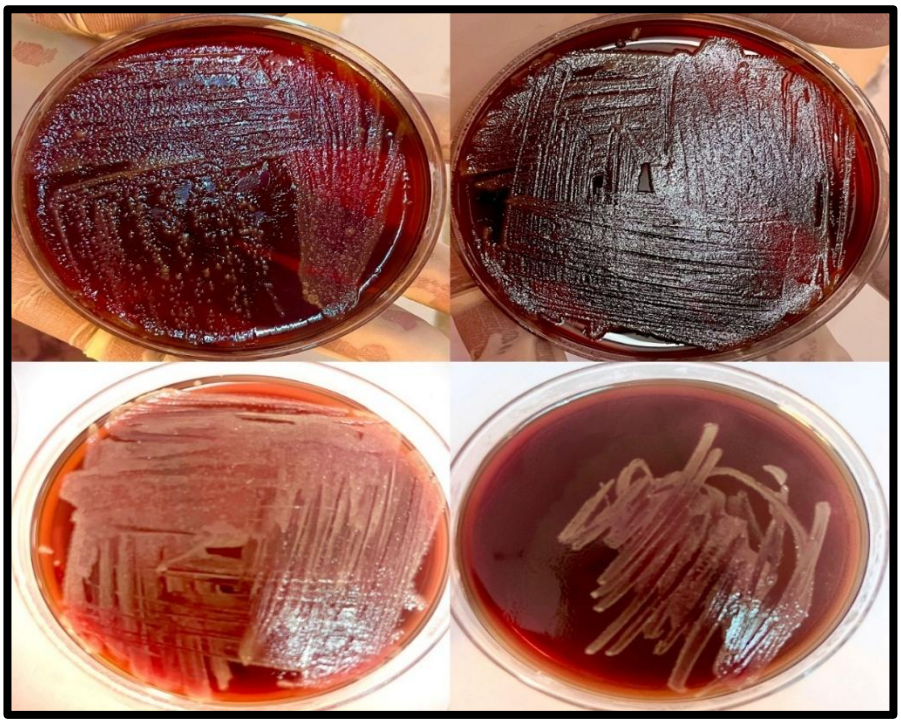

Figure 2: Alpha- and Beta-Hemolysis of *Clostridium perfringens* on Sheep Blood Agar Plates

Observations include Gram-positive rods, α and β hemolysis on blood agar, positive gelatin liquefaction, and lecithinase activity. Additionally, these isolates demonstrated stormy fermentation in litmus milk and acid and gas production with

black precipitate in TSI agar, consistent with *C. perfringens* identification. Out of the 63 samples analyzed, 5 tested positives for *Clostridium perfringens*, resulting in an overall positivity rate of **7.94%**.

Table 2.1 Biochemical, Morphological, and Phenotypic Characteristics of *Clostridium perfringens* Isolated from Poultry Dropping-Contaminated Soil Samples (PDCS 1 to 39)

Sample Code	**PDCS 1-11**	**PDCS 12**	**PDCS 13-20**	**PDCS 21**	**PDCS 22-30**	**PDCS 31-39**
Location	Ratlam	**Ratlam**	Mandsaur	**Mandsaur**	Mandsaur	Ujjain
Gram Staining	G-	**G+**	**G-**	**G+**	G-	G-
Motility Test	-	**—**	-	**—**	-	-
Spore Staining	-	**;**	-	**;**	-	-
Hemolysis on Blood Agar	-	**α β**	-	**α β**	-	-
Gelatin Liquefaction Test	-	+	-	+	-	-
Litmus Milk Fermentation Test	-	**	-	**	-	-
Nitrate Reduction Test	-	+	-	+	-	-
Urease Test	-	-	-	-	-	-
Cytochrome Oxidase Test	-	-	-	-	-	-
Catalase Test	-	-	-	-	-	-
Indole Production Test	-	-	-	-	-	-
H_2S Production Test	-	+	-	+	-	-
Simmon's Citrate Test	-	+	-	+	-	-
TSI Agar Test	-	##	-	##	-	-
DNase Enzyme Activity Test	-	+	-	+	-	-
Lecithinase Enzyme Activity	-	+	-	+	-	-
Result	-	**Positive**	-	**Positive**	-	-

Table 2.2 Biochemical, Morphological, and Phenotypic Characteristics of *Clostridium perfringens* Isolated from Poultry Dropping-Contaminated Soil Samples (PDCS 40 to 63)

Sample Code	**PDCS 40-44**	**PDCS 45**	**PDCS 46-49**	**PDCS 50**	**PDCS 51-62**	**PDCS 63**
Location	Indore	**Indore**	Indore	**Indore**	Indore	**Indore**
Gram Staining	G-	**G+**	G-	**G+**	G-	**G+**
Motility Test	-	**—**	-	**—**	-	**—**
Spore Staining	-	**;**	-	**;**	-	**;**
Hemolysis on Blood Agar	-	**α β**	-	**α β**	-	**α β**
Gelatin Liquefaction Test	-	+	-	+	-	+
Litmus Milk Fermentation Test	-	**	-	**	-	**
Nitrate Reduction Test	+	+	-	+	-	+
Urease Test	-	-	-	-	-	-
Cytochrome Oxidase Test	-	-	-	-	-	-
Catalase Test	-	-	-	-	-	-

Indole Production Test	+	-	-	-	-	-
H_2S Production Test	-	+	-	+	-	+
Simmon's Citrate Test	-	+	-	+	-	+
TSI Agar Test		##	-	##	-	##
DNase Enzyme Activity Test	-	+	-	+	-	+
Lecithinase Enzyme Activity	-	+	-	+	-	+
Result	-	**Positive**	-	**Positive**	-	**Positive**

Table 2.1 & 2.2 Note: PDC = Poultry Droppings-Contaminated Soils; + = Positive; − = Negative; ** = Stormy fermentation; * = No fermentation; ## = Acid and gas production with black precipitate; # = No reaction; α = Alpha hemolysis; β = Beta hemolysis; G+ = Gram-positive rod-shaped bacteria; G± = Gram-variable rod-shaped bacteria; ; = Subterminal spores; __ = non-motile

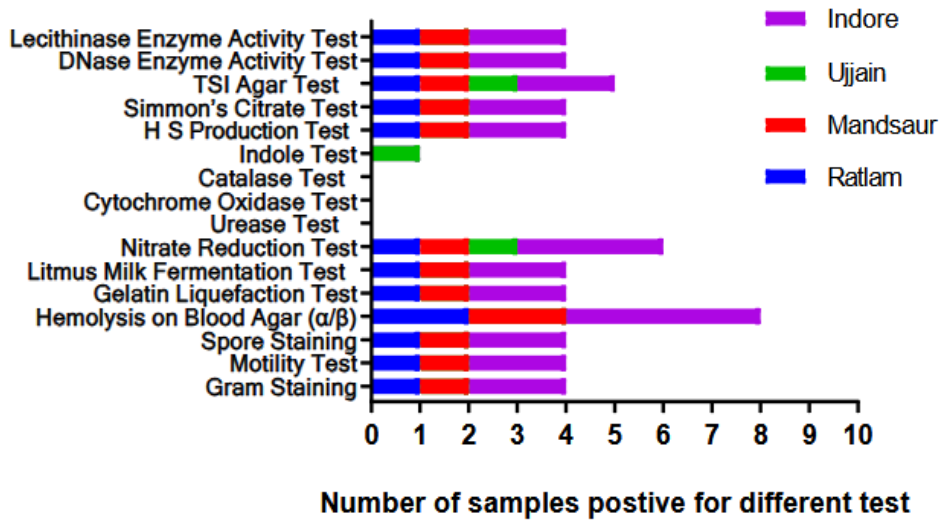

Figure 3: Stacked Graph Showing the Number of Poultry Droppings-Contaminated Soil Samples Testing Positive for Different Morphological, Biochemical, and Phenotypic Characteristics of *Clostridium perfringens.*

Effect of Environmental Factors on the Growth of *Clostridium Perfringens*-Positive Samples Isolated from Poultry Dropping-Contaminated Soils

1. Humidity: - Humidity is a critical environmental parameter that influences the moisture availability for microbial growth. The study examined the influence of environmental humidity on the growth of *Clostridium perfringens* by incubating cultures in CPSB under varying humidity levels of 50%, 70%, and 90% RH. Growth was monitored over a 48-hour period by measuring the optical density (OD600) at different time points. The results demonstrated that humidity significantly affected the growth rate and overall biomass production of *C. perfringens*. At **50% humidity**, bacterial growth was significantly lower compared to higher humidity conditions. The OD600 values increased gradually over time, reaching a peak at approximately 36 hours, after which growth plateaued. The initial lag phase was more pronounced at this humidity level, suggesting that lower moisture availability may have delayed bacterial adaptation and division. The maximum OD600 value remained lower than that observed under 70% and 90% humidity conditions, indicating that reduced humidity may limit the overall growth potential of *C. perfringens*. At **70% humidity**, *C. perfringens* exhibited a faster growth rate, with OD600 values rising more rapidly during the exponential phase. The lag phase was shorter compared to 50% humidity, and the culture reached its peak OD600 around 30 hours, indicating an earlier transition into the stationary phase. This suggests that moderate humidity levels provide an optimal environment for bacterial proliferation by maintaining adequate moisture for metabolic activity and nutrient absorption. The growth curve showed a steady increase without significant delays, highlighting the favorable conditions provided at 70% RH. At **90% humidity**, bacterial growth was the highest among the tested conditions. The OD600 values increased sharply, reaching peak biomass around 24 hours, demonstrating an even shorter lag phase compared to 70% humidity. The rapid growth

suggests that high humidity levels enhance bacterial metabolism and division, potentially by preventing desiccation and maintaining optimal hydration of cells. However, after 24 hours, the growth curve showed signs of an early decline, which could be due to nutrient depletion or the accumulation of metabolic byproducts. The results indicate that while high humidity supports rapid bacterial growth, prolonged exposure might lead to environmental stress or resource exhaustion. The comparative growth curves indicate a direct correlation between humidity and bacterial proliferation, with higher humidity levels promoting faster and more extensive growth of *Clostridium perfringens*. The variation in lag phase duration across the different humidity conditions suggests that moisture availability is crucial for bacterial adaptation and division rates. Additionally, the final OD600 values at each humidity level confirm that the total biomass yield was highest at 90% RH, followed by 70%, and then 50%, as shown in **Figure 4** Growth Curve of *Clostridium perfringens* at Different Relative Humidity Level**.**

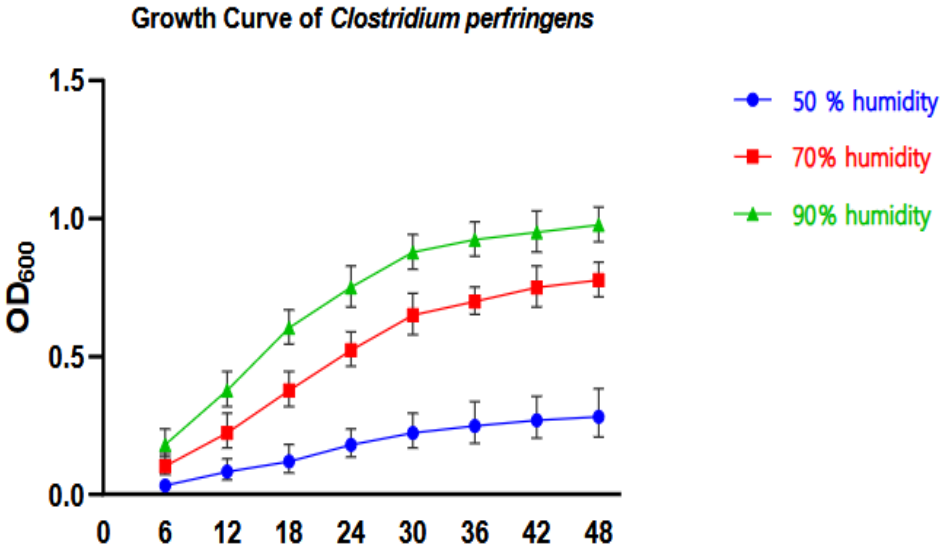

Figure 4: Growth Curve of *Clostridium perfringens* at Different Relative Humidity Levels

2. Incubation: - As an obligate anaerobe, *Clostridium perfringens* thrives in oxygen-free environments. Two sets of incubation conditions—strictly anaerobic and microaerophilic—were compared to evaluate the growth of the isolates.
Strictly Anaerobic Conditions: The isolates showed 70% of their maximum growth under strictly anaerobic conditions. Anaerobic jars equipped with gas-generating sachets were used to create an oxygen-free environment. This result highlights the bacterium's inability to tolerate oxygen, consistent with its physiological requirements.

Microaerophilic Conditions: Under microaerophilic conditions, growth was reduced to 30%. Although the bacterium exhibited some tolerance to low levels of oxygen, its metabolic activity and replication were significantly inhibited.

The results highlight the critical role of strictly anaerobic environments in supporting *C. perfringens* growth. This finding is particularly relevant for laboratory studies and field conditions, where maintaining anaerobic conditions is essential for isolating and cultivating the bacterium.

3. Temperature and pH:-

The study assessed the impact of temperature and pH on *Clostridium perfringens* growth by analyzing optical density (OD600) values over 48 hours. The results showed significant variations in bacterial proliferation depending on temperature and pH conditions.

At different **temperature conditions**, growth was slowest at **20°C**, with OD600 values increasing gradually, indicating a prolonged lag phase and reduced metabolic activity. Growth improved at **25°C**, but the exponential phase was delayed compared to higher temperatures. The optimal growth was observed at **35°C**, where OD600 values increased rapidly, reaching the highest peak among all tested conditions. At **40°C**, growth remained high but slightly lower than at 35°C, suggesting possible stress at higher temperatures. At **45°C**, growth declined significantly, indicating that elevated temperatures may negatively impact bacterial metabolism or viability. These results suggest that *C. perfringens* thrives best between 30°C and 40°C, with 35°C being the most favorable temperature for growth, as **shown in Figure 5**

The pH experiment revealed that the growth of *C. perfringens* is highly dependent on pH levels. At pH 3.0, no significant growth was observed, indicating that acidic conditions inhibit bacterial proliferation. At pH 5.0, minimal growth occurred,

suggesting partial adaptation to slightly acidic conditions, though with limited metabolic activity. The highest growth was recorded at pH 7.0, confirming that neutral pH is optimal for *C. perfringens* proliferation. At pH 9.0, growth was slightly lower than at neutral pH, indicating tolerance to mild alkalinity. However, at pH 11.0, growth was significantly reduced, demonstrating that highly alkaline condition are unfavourable, as shown in **Figure 6**.

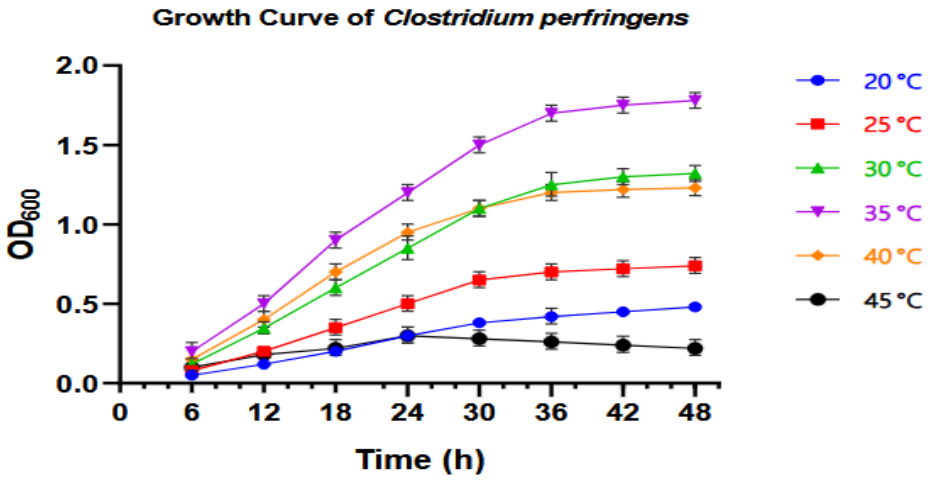

Figure 5: Effect of Temperature on the Growth of *Clostridium perfringens*

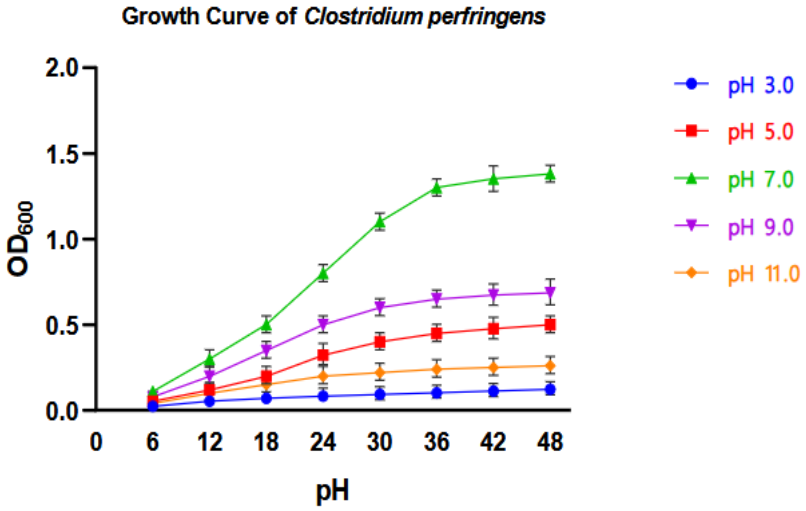

Figure 6: Effect of pH on the Growth of *Clostridium perfringens*

4. Antibiotics: - The study assessed the susceptibility of *Clostridium perfringens* to various antibiotics using the disc diffusion method. The results showed significant variations in the effectiveness of different antibiotics based on the measured zones of inhibition. The antibiotics tested included Clindamycin, Rifampin, Tetracycline, Amoxicillin, Erythromycin, Chloramphenicol, Ciprofloxacin, Gentamicin, and Vancomycin. The inhibitory effect of each antibiotic was determined by measuring the diameter of the zone of inhibition, which indicates the extent to which bacterial growth was suppressed. Among all the tested antibiotics, **Ciprofloxacin exhibited the largest zone of inhibition**, demonstrating strong antibacterial activity against *C. perfringens*. This suggests that Ciprofloxacin, a fluoroquinolone antibiotic, is highly effective in disrupting bacterial DNA replication and inhibiting bacterial growth. **Rifampin and Chloramphenicol also showed significant inhibition**, with zones of inhibition slightly smaller than Ciprofloxacin but still indicative of strong antibacterial action. Rifampin, a rifamycin-class antibiotic, targets bacterial RNA synthesis, which may explain its effectiveness against *C. perfringens*. Similarly, Chloramphenicol, which inhibits protein synthesis, displayed substantial antibacterial activity, making it a viable option for controlling *C. perfringens*-related infections. Moderate inhibition was observed with **Tetracycline, Erythromycin, and Clindamycin**, which produced smaller inhibition zones than Ciprofloxacin, Rifampin, and Chloramphenicol. Tetracycline, a broad-spectrum antibiotic that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit, exhibited a moderate inhibitory effect, indicating partial susceptibility

of *C. perfringens*. Erythromycin, a macrolide antibiotic that targets the 50S ribosomal subunit, showed a similar inhibition pattern, suggesting that *C. perfringens* may possess some level of intrinsic or acquired resistance to macrolides. Clindamycin, which also targets the 50S ribosomal subunit, displayed comparable inhibition to Erythromycin, reinforcing the idea that *C. perfringens* has variable susceptibility to macrolide and lincosamide antibiotics. In contrast, **Amoxicillin, Gentamicin, and Vancomycin exhibited the weakest inhibitory effects**, producing minimal or negligible zones of inhibition. Amoxicillin, a beta-lactam antibiotic, showed very little inhibition, indicating that *C. perfringens* is highly resistant to beta-lactams. This resistance may be due to the production of beta-lactamase enzymes that degrade the antibiotic before it can exert its antibacterial effects. Gentamicin, an aminoglycoside that disrupts protein synthesis by binding to the 30S ribosomal subunit, also had minimal effectiveness against *C. perfringens*, suggesting that this bacterium may possess intrinsic resistance to aminoglycosides. Vancomycin, a glycopeptide antibiotic that inhibits cell wall synthesis, was largely ineffective, confirming that *C. perfringens* is resistant to glycopeptides. The results suggest that while some antibiotics, such as Ciprofloxacin, Rifampin, and Chloramphenicol, are highly effective against *C. perfringens*, others, including Amoxicillin, Gentamicin, and Vancomycin, show limited or no efficacy. These findings are critical in guiding antibiotic selection for treating *C. perfringens*-associated infections. The moderate inhibition observed with Tetracycline, Erythromycin, and Clindamycin suggests that these antibiotics may be effective in certain cases but may require higher doses or combination therapy to achieve optimal bacterial suppression.The resistance patterns observed in this study highlight the need for antibiotic stewardship to prevent the emergence of multidrug-resistant *C. perfringens* strains. The high efficacy of Ciprofloxacin and Rifampin suggests that fluoroquinolones and rifamycins may be preferred choices for treating *C. perfringens* infections. However, the observed resistance to beta-lactams and glycopeptides underscores the importance of routine antibiotic susceptibility testing before prescribing antibiotics for *C. perfringens* infections. In conclusion, the study demonstrated that *C. perfringens* exhibits varying degrees of susceptibility to different antibiotics. Ciprofloxacin, Rifampin, and Chloramphenicol were the most effective, while Amoxicillin, Gentamicin, and Vancomycin were the least effective. The results emphasize the need for targeted antibiotic therapy based on susceptibility testing to ensure effective treatment and reduce the risk of antibiotic resistance in *C. perfringens*, as shown in **Figure 8**: Heatmap Showing Antibiotic Susceptibility of *Clostridium perfringens* Based on Zone of Inhibition and **Figure 7**

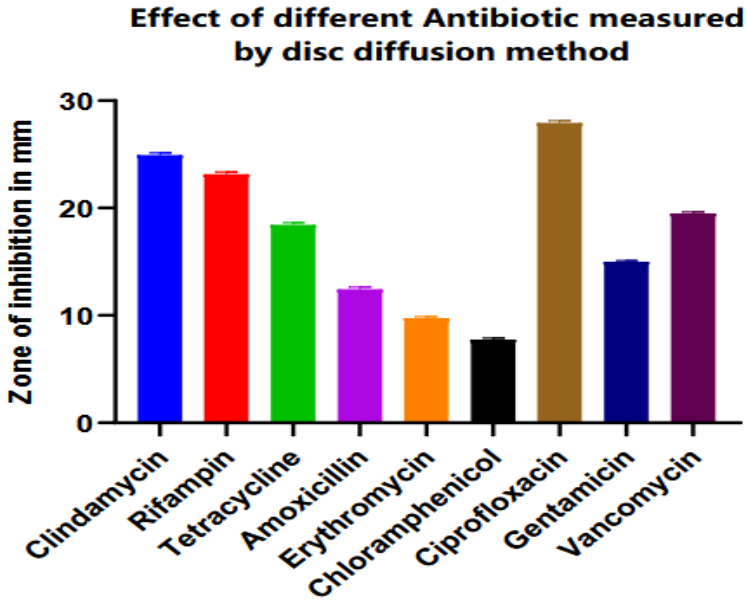

Figure 7: Antibiotic Susceptibility Profile of *Clostridium perfringens*.

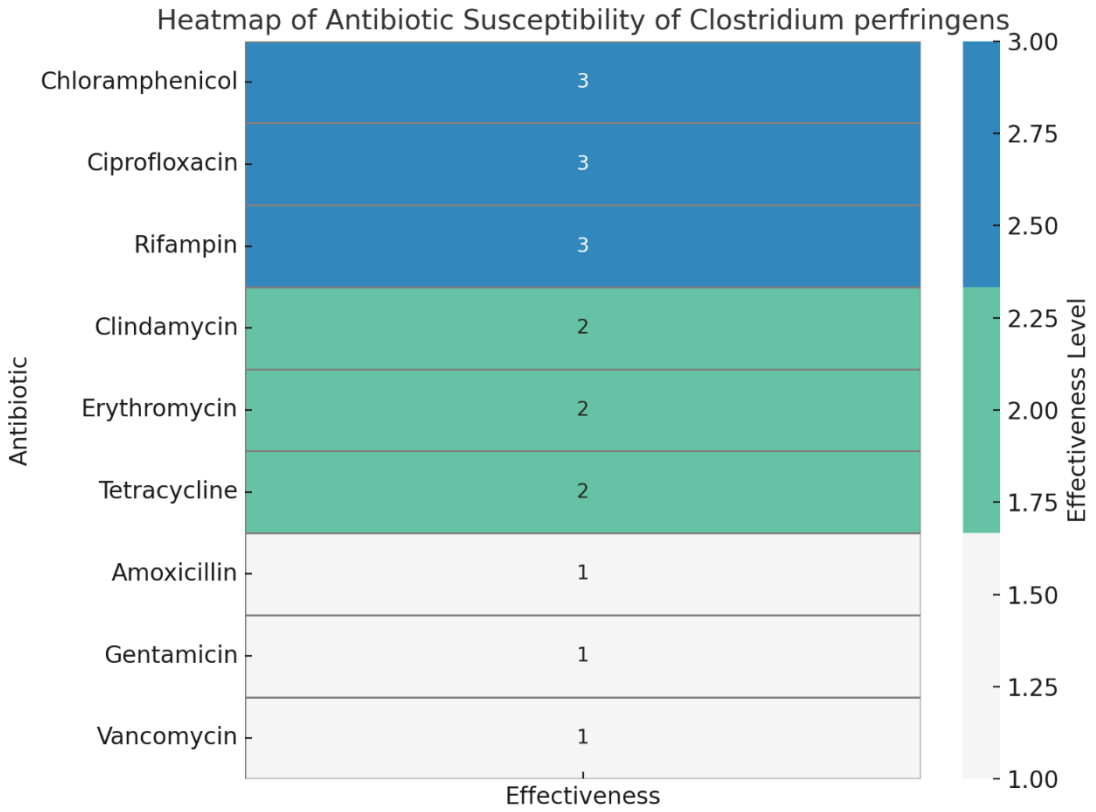

Figure 8: Heatmap Showing Antibiotic Susceptibility of *Clostridium perfringens* Based on Zone of Inhibition

4. DISCUSSION

The findings of this study highlight the ecological adaptability of *Clostridium perfringens* in poultry farm environments. Among the 63 poultry droppings-contaminated soil samples collected, five isolates (7.94%) tested positive for *C. perfringens*, indicating that the bacterium can persist in specific environmental niches enriched with organic matter and suitable anaerobic conditions (Van Immerseel et al., 2004). Environmental variables such as humidity, temperature, and pH were found to significantly influence bacterial proliferation. The highest growth was observed at 35°C and pH 7.0, conditions previously reported as optimal for the growth and sporulation of *C. perfringens* (Medical, 1981; Mehdizadeh Gohari et al., 2021). Growth at 90% relative humidity (RH) was significantly enhanced, suggesting that moisture-rich conditions found in poultry waste-enriched soils promote bacterial metabolism and resilience. Similar moisture-dependence was reported by Araujo et al. (n.d.), who emphasized the role of environmental humidity in facilitating spore viability. The bacterium's inability to grow effectively under microaerophilic conditions reaffirms its classification as an obligate anaerobe (Stevens et al., 1987). While some tolerance to low oxygen levels was noted, significant inhibition occurred under non-anaerobic conditions, which is consistent with previous findings (Tsiouris et al., 2018). The susceptibility of isolates to antibiotics revealed strong inhibition by Ciprofloxacin, Rifampin, and Chloramphenicol. These results are in agreement with findings by Chen et al. (2024), who reported high efficacy of fluoroquinolones and rifamycins against *C. perfringens*. Conversely, marked resistance to Amoxicillin, Gentamicin, and Vancomycin indicates possible widespread misuse or overuse of these antibiotics in poultry production, potentially leading to resistant strains (Asha et al., 2006). This reinforces the need for targeted antimicrobial use and regular resistance surveillance in poultry farms, as also suggested by Rood et al. (1978). The ability of *C. perfringens* to form spores under adverse environmental conditions such as desiccation, variable pH, and fluctuating temperatures raises concerns regarding its long-term persistence in soil ecosystems (Keyburn et al., 2008). Its potential transmission through the soil-to-crop food chain poses a significant risk to both poultry health and human food safety (de Mesquita Souza Saraiva et al., 2022; Hutchison et al., n.d.). This highlights the need for effective poultry waste management, environmental monitoring, and antibiotic stewardship to mitigate the risks posed by *C. perfringens* in agricultural systems.

5. CONCLUSION

This study demonstrates that environmental factors—particularly humidity, pH, temperature, and oxygen availability—play a pivotal role in the survival, growth, and sporulation of *Clostridium perfringens* in poultry droppings-contaminated soils. The optimal conditions identified (90% RH, 35°C, and pH 7.0 under anaerobic environments) explain the bacterium's persistence in poultry farms and its potential to cause recurrent infections. Moreover, the detection of resistance to commonly used antibiotics calls for urgent implementation of controlled antibiotic usage and routine

monitoring. Overall, these findings emphasize the need for integrated strategies involving environmental management, antimicrobial stewardship, and strict biosecurity measures to prevent the spread of *C. perfringens* and safeguard poultry health and food safety.

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