Comparison of the effect of garlic with the effect of antibiotics on the survival and growth of broiler chickens

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIA FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Olia Najon Tuson

Student ID: 15136032

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Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

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DECLARATION

I hereby solemnly declare that the research work titled "**Comparison of the effect of garlic with the effect of antibiotics on the survival and growth of broiler chickens**" submitted by the undersigned has been carried out under the supervision of Dr. M. Mahboob Hossain, Professor Microbiology Program, Department of Mathematics and Natural Sciences BRAC University, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original work. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

(Olia Najon Tuson)

Candidate

Certified

(Dr. M. Mahboob Hossain)

Supervisor

Professor

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University, Dhaka

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Abstract

The aim of the present study was to compare the effect of garlic as feed additives with the effect of antibiotics on the survival and growth of broiler chickens. The massive use of the antimicrobial drug in poultry industries is one of the reasons for the emergence of drug-resistant microbes. Transmission of resistant pathogens from animals to humans can take place through a variety of routes, such as via direct consumption, direct contact of chickens to humans or from the manure which is later used as a fertilizer or feeds for the fish.

First of all, to conduct this study chickens were divided into four groups (T1, T2, T3, and T4). Each group consisted of 200 chickens (except T4). They were given the same poultry feed. T1 was given antibiotics and other manufactured growth promoters, T2 was given garlic, T3 was given both garlic and yogurt as feed additives and T4 was given none (only regular poultry feed: starter, grower, and finisher). T4 was taken as a control group for comparison purpose. To analyze the methods and its effectiveness, results were collected carefully; data were made on the chicken's mortality rates, body weights, biochemical tests and antibiogram test of the feces samples collected from each group of chickens. Broiler chickens are extremely fast growing, they have been processed to grow and then slaughtered in 30-32 days. For this research purpose chickens were under observation for 30 days.

In the present study, it was observed that the stool samples collected from all the groups were contaminated by bacteria from Enterobacteriaceae family (*E.coli, Proteus vulgaris, Salmonellatyphi, Salmonella choleraesuis, Salmonella paratyphi* A). All of the bacterial isolates were multi-drug resistant. The survival rate and the body weights of the T2 group were higher than the T1 group. Though group T4 had only 25 chickens but they all survived. The findings showed a possibility of using garlic as a prophylactic agent. Also, few physical abnormalities were observed in the chickens from T1 group which could indicate the negative impacts of chemically synthesized drugs.

Chapter 01.
Introduction

Introduction

The modern chicken, another way of naming them is the broiler chicken, that ends up on most dinner tables today have been carefully selected and bred to produce chicken that will best thrive in today's poultry facilities. As a result this selective birds are much healthier and faster growing as they are predicted so. Although they are carefully selected for the farm but they are not totally safe from diseases and other deadly infections. However with better breeding and better living conditions such as 24 hour access to clean the room, providing clean feeds, growth promoters , feed additives and clean water could determine the safeness of these chickens. In the past, antibiotics were the most routinely used feed additives.Growth enhancement with antibiotics (sulphonamides) was first observed by Moore et al. (1946).Administering antibiotics to poultry, cattle and pigs in their feed or drinking water has had a major impact on the commercial production of meat for human consumption since the early 1950s (Jukes,1972).

However, the usage of antibiotics is not only limited but their use in livestock and poultry industry also have been banned in many countries due to the reasons like alteration of natural gut microbiota and drug resistance in bacteria and humans. As a result, to replace them without adversely affecting the performance of birds, natural growth promoters such as prebiotics, probiotics, synbiotics, enzymes, plant extracts, etc, can be used to feed the broilers (Demir et al., 2003). Phytogenic feed additives or plant extracts used as growth promoters have shown promising effect with regard to weight gain, feed efficiency, lowered mortality and increased livability in poultry (Ansari et al., 2008)

Garlic (*Allium sativum*) can be used as potential alternatives for common artificial growth promoters like antibiotics. It has been used as a spice and a native medicine for many years. It has possessed antibacterial, antifungal, antiparasitic, antiviral, antioxidant, anticholesteremic, anti-cancerous, and vasodilator characteristics (Hanieh H et al., 2010). Garlic in broiler chicken diets have been recognized for their strong stimulating effect on the immune and digestive systems in birds. Recent research works on garlic as feed additives have shown encouraging results in regards to weight gain, feed efficiency, lowered mortality and increased livability in poultry birds. Its positive effect on several cardiovascular risk factors have been also confirmed (Varmaghany et al., 2015). The use of garlic as a feed additive in broiler diets has been shown to improve feed conversion ratios and to reduce mortality (Javandel et al., 2008). As an herbal supplement, garlic

is less complicated feed additives than commercially manufactured antibiotic drugs. For this research purpose garlic was chosen to play vital role to fight against harmful pathogens andalso to maintain a healthy micro-flora in the guts of the chickens. The main purpose of this research study was to apply simplified method to treat broiler chicken from common infections rather than feeding them complicated chemical substances. Our mother nature provides us so many natural remedies. Garlic is very popular for the antibacterial properties. Garlic is well known for their powerful pungent flavor which is the main substance with medicinal properties. Garlic also contains sulfur compounds which boost the immune system, improve blood circulation. The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which can affect essential metabolism of cysteine proteinase activity involved in the virulence of *E. histolytica* (Nordqvist, 2017).

Animal gastrointestinal tracts are populated by hundreds of different species of bacteria in mind-boggling numbers. The gastrointestinal (GI) tract of poultry is densely populated with microorganisms which closely and intensively interact with the host and ingested feed. Species like Salmonella, Escherichia and streptococcus are mostly found in animals and livestock. Salmonella is a zoonotic pathogen which can readily pass from animal to humans through the consumption of contaminated meat, animal products or other food products after contamination with animal fecal material. Salmonellosis can also be acquired through direct or indirect contact with colonized animals as well as through consumption of contaminated water. Salmonellae can also be considered a common commensal of the gut microflora of animals, including mammals, birds, reptiles, amphibians, fish, and shellfish. Fecal contamination is the main source of food and water contamination playing a large role in the dissemination of salmonellae in the environment and subsequently the food supply chain. Meat animals can be infected and act as reservoirs of salmonella (Garland et al., 2006).The ability of *Salmonella* species to cause infection involves attachment and colonization of intestinal columnar epithelial cells (Gill et al., 1995). In severe cases, infection may progress to septicemia and death (Klochko, 2011). The prevalence of *Salmonella enterica* associated with raw poultry and poultry meat products have been well-documented (Blackburn et al., 2002).

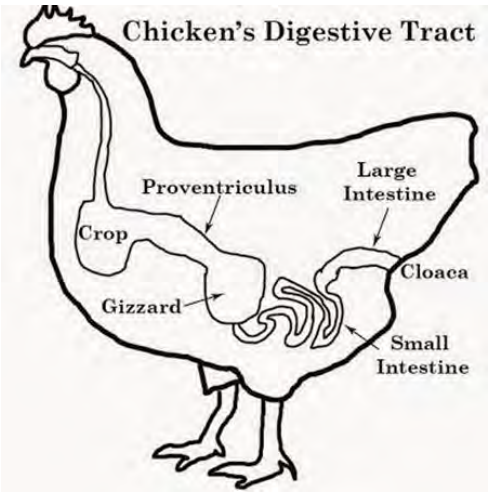


Figure: A chicken's digestive tract where bacteria harbored.

The gut microbiome benefits the host by providing nutrients from otherwise poorly utilized dietary substrates and modulating the development and function of the digestive and immune system. In return, the host provides a permissive habitat and nutrients for bacterial colonization and growth. Gut microbiome can be affected by diet, and different dietary interventions are used by poultry producers to enhance bird growth and reduce risk of enteric infection by pathogens. There also exist extensive interactions among members of the gut microbiome. A comprehensive understanding of these interactions will help develop new dietary or managerial interventions that can enhance bird growth, maximize host feed utilization, and protect birds from enteric diseases caused by pathogenic bacteria (Pan& Yu, 2013).

Most of the bacteria in an animal's gut are not harmful. However, even seemingly healthy animals will have some harmful bacteria such as *E.coli*, or *Salmonella* in their guts. However, the bad bacteria are in such small quantities that they can't do any harm. The populations of these bad bacteria are held in check by the good bacteria that out-compete them for resources and keep their numbers so low they can't make the animal sick. To maintain this balance probiotics could play a vital rule. With this goal, probiotic was also introduced in this research and fed to chicken when they were over 14 days old.In broiler nutrition, probiotic species belonging to*lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida* and

Saccharomyces have a beneficial effect on broiler performance, modulation of intestinal micro-flora and pathogen inhibition and promoting microbiological meat quality of broilers. The mode of action of probiotics in poultry includes maintaining normal intestinal microflora by competitive exclusion antagonism, lowering the pH through acid fermentation, competing for mucosal attachment and nutrients, producing bacteriocins, stimulating the immune system associated with the gut, increasing the production of short-chain fatty acids (Sarangi, 2016). In this study, yogurt was used as a probiotic supplement for broiler chickens. A moderate amount of yogurt was consumed by chickens with drinking water.

Antibiotics play a vital role in poultry business. Manufacturer companies release hundreds of varieties antimicrobial medicines to fight against these common entities. But they sometime adversely affect the natural system, especially to chicken as well as the consumer of the broiler chickens by interrupting their natural gut flora. Chemical antibiotics which are normally given to the chickens are derived from bacteria or moulds that are toxic to other bacteria. Sometime the targeted microorganisms somehow develop resistance against the medicine and get stronger. This could lead to develop so many dangerous or mutated bacterial strains. Some bacteria are naturally resistant to certain types of antibiotics so the targeted bacteria survive and continue to multiply, causing more harm.

Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine. This acquired resistance occurs not only in pathogenic bacteria but also in the endogenous flora of exposed individuals (animals and humans) or populations. Therefore the antibiotic selection pressure for resistance in bacteria in poultry is high and consequently their fecal flora contains a relatively high proportion of resistant bacteria (Suzanne, 1979). In this study the activity of antibiotic growth promoter was also determined and observed. The main concern was to see the resistant level of different antibiotic drugs. In this poultry farm, amoxicillin, colistin, sulfamethoxazole were given to a one group of broiler chickens. These antibiotics improve feed conversion and body weight gain presumably by altering the composition and activities of micro flora (Knarreborg et al., 2002).This practice may modify the intestinal flora and create a selective pressure in favor of resistant bacteria. In response to the emergence of antibiotic resistance, several European countries have restricted or banned the use of antibiotics as growth promoters (Diarra et

al., 2007). *Escherichia coli* is a ubiquitous organism in the chicken gastrointestinal tract and is regarded as a major pathogen of worldwide importance in commercially produced poultry. It can cause diseases including colibacillosis and air sacculitis in poultry, resulting in significant economic losses (Tadesse et al., 2012).

A recent report on surveillance of antimicrobial resistance (WHO Antimicrobial Resistance: Global Report on Surveillance, 2014) forewarns that a crisis in antimicrobial resistance is eminent, and the antibiotic era is at risk if remedial action is not undertaken to delay the crisis while governments and drug companies cooperate in searching for new antibiotics. It called for reduction in the use of antibiotics in human and veterinary medicine. Concerning food animals, the magnitude of transmission of resistant pathogens to humans is unknown and needs to be addressed through better data obtained by improved surveillance, and it needs to be harmonized with human data (similar to the recent Scottish Salmonella report – authors' comment). Use of antibiotics in food animals should be reduced, but therapeutic use will have to be continued to protect the food chain. Regulation of human and companion animal use was not mentioned (Ragland et al., 2014).

The main objective of this research is to avoid antibiotic drugs to treat broiler chicken and also to find alternative ways which is more convenient and cost efficient as well. So that mass people can easily understand and apply this technique.

Figure 1: Chicks at the farm with feeders.

Chapter 02.

Methods

2. Methods

2.1 Selection of area

The study was conducted during the period of september to december 2017, at first the poultry farm was selected in Narsingdi district. As broilers only live for several weeks before they are slaughtered. So these chickens were raised for 30days at this farm for this study purpose after that the desired stool samples were brought to the laboratory, department of MNS, BRAC University for analysis.

2.2. Feed and medication

The chickens were provided commercially prepared broiler starter, grower and finisher, recommended by veterinarian. The feeds were consist of crude protein, moisture, fat, crude fiber. As the chicken gets older the percentage of fat gets lower and protein gets higher of the feeds and for fighting against diseases and infections they were given antibiotics, garlic powder and yogurt. It is very important to know that these broiler chickens were provided huge amount of drinking water. The water pots were filled up regularly, three times a day, for 200 chickens, it started from 4-5 liters to 8-10 liters a day. The antibiotics which were used for this period, were colimox vet (consist amoxicillin and colistin) and sulpha cox.

Figure 2: Chickens growing up in the farm.

2.3 Preparation of chickens

Chickens were divided into four groups, and each contained 200 chickens except one group, which contained only 25 chickens. The groups were made based on their medications, feed additives and growth promoters that had been used. One group was given antibiotics as growth promoter, one group was given garlic (garlic powder), and another group was given garlic and yogurt both and the last one which consisted of 25 chickens, was not given any feed additives and supplements except regular feed and water. The last group was named control group. It was used for the comparison purpose to see how chicken acts without any medication or supplements. All these groups were provided same chicken feeds.

For research purpose these chickens were categorized into four groups and their given names were:

1. **Group T1- fed Antibiotics and other chemically synthesized drugs.**
2. **Group T2- fed garlic**
3. **Group T3- fed Garlic and yogurt.**
4. **Group T4- Control.**

- For group T1, the 200 chickens were given colimox vet, which basically contains colistin and amoxicillin along with their regular feeds. The standard dosage was prepared by mixing 0.5gm of colimox with one liter of drinking water. This medicine was given 3-5days per week. From the very first day, it was first given in 3-4 liters of water but as the chicken got older, the consumption of water got higher up to 8-10 liters per day. It was continued up to 21-22 days. Sulpha cox and Rena-c were given twice during third and fourth week, with water.

Figure 3: Drugs that used for group T1 chickens.

- For T2 group, another 200 chickens were given same feeds and habitant but now instead of antibiotics they were provided garlic. First the garlic powder was mixed with water in a moderate amount. This dosage of medication was started when the chickens were only 2 days old. First 0.5 gram of garlic powder was mixed with 1 liter of drinking water for their consumption. It was given 2 days in the first week. Then during the second week, it was given for 3days. During third week, 0.75gm garlic (0.75gm/1 liter water) was provided for 3 days. At last week, 1 gram garlic powder per liter water, was given for 2 days. This procedure was applied in 8-10 liters water depending on their water consumption rate.
- For T3 group, 200 chickens were selected and given the same feeds. They were provided same amount of garlic like T2 group, from first week to fourth week. But yogurt was also added to their diet after second week. At day14, chickens were given 10gms of yogurt per liter water. This procedure was followed 2 times in the same week. After 21 days, they were given 20gms of yogurt per liter water. Again same procedure was repeated for 2 times on that week. These procedures were applied in 8-10 liters of water depending on their water consumption rate.
- For T4, no antibiotics, garlic or yogurt were given to consume. These 25 chickens were simply raised by their regular chicken feeds and drinking water.

Table 1: Total costs for growth promoters and feed additives to treat chickens:

Chicken Group	Name of the drugs and growth promoters	Price in BDT (Bangladeshi currency)
T1	▪ Sulpha-cox (anti-coccidial) ▪ Colimox ▪ Rena-C (Vitamin c)	▪ 270 -/ ▪ 347 -/ ▪ 100-/
T2	▪ Pran Garlic powder	▪ 70 -/
T3	▪ Pran garlic powder ▪ Arong sour yogurt	▪ 70-/ ▪ 70-/
T4	▪ Not given	▪ --

2.3.1Measuring chicken's weight

For measuring the weight of chickens for the study purpose, from every group 5 chickens were randomly picked to measure their weight. So the chart shows the weight of the 5 chickens. Every week the chickens that were selected for this process, was repeated and completely picked up randomly from each 200 number of chicken group. As every group consist of 200 chickens except the control group, which only had 25 chickens.

2.3.2. Chicken mortality rate

The four groups of chickens were raised on different manner. As one group was given antibiotic, another one was given garlic, other one on yogurt and garlic both and last one was given nothing to enhance their immunity and protection from diseases. So the death rate from each group was measured very carefully to see the result of these different treatment methods and also to see which method was more effective.

2.4 Collection of stool samples

At the day 14, chicken stools were collected from T1, T2, T3 and T4 groups. From each group at least two samples were taken for laboratory test. Normal chicken droppings are firm and brown with a white part on the top. While collecting chicken stool, a very standard procedure was followed, only fresh chicken droppings were collected, so that no other contamination from air got mixed with the sample. Sterile hand gloves were worn during the sample collection, and sterilized spoon was used to collect the samples. It was collected carefully to avoid dirt. The samples were collected in falcon tubes with 9ml saline water in each tube. The sample were labelled properly

and brought directly to the laboratory where bacteriological study was conducted. The same procedure was followed during the second time. At the day 22 the chicken stools from four different groups were collected for bacteriological tests.

2.4.1. Processing of stool samples.

- The stool samples were processed by homogenizing 2-3 grams of each sample in 9ml of sterile physiological saline and then cultured in four types of agar media such as Nutrient agar (NA), MacConkey agar (Mac), eosin methylene blue agar (EMB), salmonella shigella agar (SSA).
- All the media were prepared for each chicken group.
- After autoclave the media was poured into the plates.
- All the bacterial cultures were grown in the incubator at 37 ºC for 20-24 hours.
- After studying the bacterial growth in different culture media, some specific colonies were selected and sub cultured into different selective media such as EMB and NA media from each chicken group.
- However each of the organisms from their previous culture was streaked on nutrient agar plate.
- And then prepared for biochemical tests to confirm the bacterial identification.

2.5 Biochemical tests

A series of biochemical tests such as Methyl-Red (MR), Voges-proskauer (VP), triple sugar iron (TSI), Indole, Urease (MIU), citrate utilization test and catalase test were performed according to the procedure described by Buxton and Fraser (1977), Merchant and packer (1967) and OIE (2004).

2.5.1 Methyl-Red (MR) test

- Six ml of dextrose phosphate (MR-VP) broth was prepared in eight test tubes, 3ml from each of the test tubes were transferred to another eight different empty test tubes.

- All the sixteen test tubes were labelled according to the sample being tested and test being conducted.
- All the bacterial samples to be tested were inoculated into 3ml dextrose phosphate broth (MR-VP) which contained dextrose and a phosphate buffer and incubated at 37ºC for 24 hours.
- When the incubation period was over, three drops of the methyl red reagent was added into the eight tubes labeled as MR test to check the pH.
- Development of a red colour would indicate a positive result, where a yellow colour would indicate a negative result.

2.5.2 Voges–Proskaur (VP) test

- All the bacterial samples to be tested were inoculated into 3 ml dextrose phosphate broth (MR-VP broth) which contained dextrose and a phosphate buffer and incubated at 37 ºC for 24 hours.
- After the incubation period was over, 10 drops of Barritt's reagent A was added to each of the test tubes and the cultures were shaken.
- Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
- After 15 minutes, the colours of the cultures were examined and the results were recorded. Appearance of a red colour was taken as a positive result.

2.5.3 Triple Sugar Iron (TSI) test

- TSI media was prepared into a screw capped test tube, and solidified to obtain a slant and butt at the length of the test tube.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully stabbed at the butt of the TSI (dextrose, lactose & sucrose) followed by slow streaking at the slant.
- The screw caps of the test tube were loosened and the tube was incubated at 37ºC for overnight. After the 24-48 hours of the incubation period, the test tube was examined to observe carbohydrate fermentation, Carbon dioxide (CO_2) and Hydrogen sulfide (H_2S) gas production.
- If the organism is able to ferment all the three sugars, then the butt would turn into yellow colour indicating the production of acid and the subsequent decrease in the pH of the media,

whereas a red colour in the slant and butt indicated that the organism being tested is a non-fermenter and the media remains alkaline.

- Presence of bubbles, splitting and cracking of the medium is the indication of CO_2gas production. A black precipitation in the butt of the tube is the indication of H_2S production.

2.5.4 Urease (MIU) test

- The broth medium was inoculated with a loopfull of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.
- The cap was left a bit loose and incubated the test tubes at 35 °C in ambient air for 18 to 24 hours.
- Cultural characteristics observed with added 40% Urea solution (FD048) after incubation at 35-37°C for 18 - 24 hours.
- After obtaining the tubes from the incubator, the tubes were observed for color changes to interpret the result carefully.

2.5.5 Citrate test

- Eight small vials were taken, into which slant of the Simmon's citrate agar was prepared and allowed to solidify.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully streaked onto the slope of the citrate media.
- The vials were incubated at 37ºC for 24 hours.
- Over these 24 hours, if the organism had the ability to utilize citrate, it would change the color of the media from deep green Prussian blue. A negative result would keep the color unchanged.

2.5.6 Indole test

- Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products – one of which is indole.
- Eight sterilized test tubes containing tryptophan broth were taken for this test.
- The tubes were inoculated the aseptically by taking the growth from 18 to 24 hours culture.
- The tubes were incubated at 37°C for 24-28 hours.
- 0.5 ml of Kovac's reagent was added to the broth culture.
- And then it was observed for the presence or absence of ring.
- For positive reaction**:** The development of a blue color within 3 minutes. and for negative reaction**:** The development of a pink color within 3 minutes

2.5.7 Catalase test

- A number of autoclaved glass slides were taken, and a drop of the catalase reagent (Hydrogen peroxide) was placed on each of the glass slides.
- The glass slides were labeled according to the sample being tested.
- A colony for each of the bacteria to be tested was taken from a nutrient agar plate, and later placed onto the reagent drops on each of the glass slides.
- An immediate bubble formation indicated a positive result.
- The same procedure was carried out for rest of the organisms.

2.6 preparation of Stock sample

For short-term preservation, 2 ml of T1N1 agar butt in a vial was inoculated by stabbing bacterial growth of each isolate from nutrient agar plate. Then the vial was kept at 4 ºC for an hour to gelatinize. After an hour, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

2.7 Methods for Detection of antibacterial activity

Using a sterile inoculating loop, one or two colonies of the organism to be tested were taken from the subculture plate. The organism was suspended in 8ml of physiological saline. The test tube containing the saline was then vortexed to create an overall smooth suspension. There were eight different samples from four different groups of chicken. So total eight test tubes with physiological saline were prepared from eight different sub-cultured samples

2.7.1 Comparing with the McFarland Solution

McFarland solution is an essential material needed before testing the microorganisms for their sensitivity. McFarland standards are used as reference to adjust the turbidity of any given bacterial suspension. This is done to make sure that the number of bacteria is within a given range to standardize the microbial testing. This would also help avoid any error in result, because if the suspension is too heavy or too diluted, an error might occur for any given antimicrobial agent.

- The bacterial suspension prepared was compared with the commercially available McFarland solution 2 (for detection of inhibition rate) and McFarland solution 0.5 (for detection of zone of inhibition by agar disc/well diffusion method). A bacterial suspension which matches with McFarland 2 is supposed to contain 6×10^8 colonies per ml. A bacterial suspension which matches with McFarland 2 is supposed to contain 1.5×10^8 colonies per ml(McFarland, 1907).

2.7.2 Procedure of dilution

- Bacterial suspension matched with McFarland 2 was subjected to 10 fold dilution in saline.
- Saline was taken separately in sets of 8 tubes.
- And bacterial inoculation from each sub cultured plate was taken to dilute with saline.
- Rate of inhibition in case of every diluted tube was then calculated and averaged to detect actual inhibition rate.

2.7.3 Preparation of Mueller Hinton Agar (MHA)

For antibiogram test, diluted samples from physiological saline tubes were cultured in MHA media. MHA media was autoclaved and then prepared to make a culture plate media.

2.7.4 Selection of Antibiotics

Table 2: List of antibiotics which were selected for the antibiogram test and their zone ranges

Antibiotic's name	Disc code	Diameter of Zone of Inhibition in mm		
		Resistant or less	Intermediate	Sensitive or more
Amoxicillin.	AMX 30	13	14-17	18
Ceftazidime.	CAZ 30	14	15-17	18
Ceftriaxone.	CRO 30	13	14-20	21
Cefeprime	CPM 30	14	15-17	18
ciprofloxacin	CIP 5	15	16-20	21
Colistin	CL 10	10	-	11
Doxycycline	DO 30	12	13-15	16
Gentamicin	GEN 10	12	13-14	15
Levofloxacin.	LE 5	13	14-16	17
Piperacillin/ Tazobactam	TZP 110	17	18-20	21
Metronidazole.	MTZ 50	13	14-15	16
Moxifloxacin.	MXF 5	20	21-23	24
Nitrofurantoin.	F 50	14	15-16	17
Sulfamethoxazole.	COT 25	10	11-15	16

2.7.5 Agar disc and well diffusion method

When a filter paper disc containing a chemical is placed on the agar the chemical will diffuse from the disc or well into the agar. This diffusion will place the chemical in the agar only around the disc and around the well. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition". Many conditions can affect a disc diffusion susceptibility test. When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions that must be constant from test to test include the

agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature, and atmosphere). The amount of organism used is standardized using McFarland 0.5 standard for this method.

2.7.6. Lawn culture of bacterial suspension on MHA media

1. A sterile swab was dipped into the bacterial suspension and the test organisms were suspended in 5 ml of nutrient broth.
2. The swab was rotated against the side of the tube using firm pressure, to remove excess fluid, but the swab was not dripped wet.
3. The dried surface of the Mueller Hinton Agar plate was used for lawn culture with the swab two times over the entire agar surface; the plate was rotated approximately 90 degrees each time to ensure an even distribution of the inoculums.
4. The plate was rimmed with the swab to pick up any excess liquid.
5. leaving the lid slightly ajar, the plate was allowed to sit at room temperature at least 3 to
6. 5 minutes for the surface of the agar plate to dry before proceeding to the next step.

2.7.7 Placement of the antibiotic disc

1. Antibiotic discs were placed on the surface of an agar plate, using a forceps, one at a time. The forceps was sterilized by immersing the forceps in alcohol. It was then burnt. The disks were gently pressed with the forceps to ensure complete contact with the agar surface. The disks were placed away from the edge of the plates so that it is easily measured.
2. Sample from each chicken group, was went for this test.
3. Every chicken group had two types of sample, so the plates were labelled based on the type of bacterial isolation. For example, bacteria isolated from garlic group named as garlic (white) for white color bacterial sample and garlic (transparent) for transparent or water color sample.
4. Once all disks are in place, the plates were inverted, and placed them in a 37 °C incubator for 24 hour.

2.7.8 Measuring the zone size

- Following incubation, the zone sizes were measured precisely using a ruler.
- All measurements were made while viewing the back of the petri dish.
- The zone size was recorded on the recording sheet

2.7.9 Data analysis

Data were analyzed using Microsoft excel version 2010.

Chapter 03.

Results

3.1 Chicken management and growth: Chickens from 4 different groups and the results after applying different feed additives and growth promoters.

3.1.1 Arrival of the chicks at the farm: At first day, these chicks were brought to the firm, provided sugar water to drink. This was done to ensure that they remained healthy and energized before they were brought into the farm as subjects. Then the chicks were divided into groups for study purposes.

Figure 4: Chicks were just brought into the farm, at the very first day.

3.1.2 Different chicken-groups:

Figure 5: Chickens from T1 group. This group was given antibiotics and other chemically synthesized drugs as growth promoters and for infection prevention.

Figure 6: Chickens from T2 group.This group was given garlic as a growth promoter and fed additive.

Figure 7: Chickens from group T3. This group was given both garlic and yogurt

Figure 8: Group T4 chickens.This is the control goup, where few chickens were selected and no medications, no fed additives and no growth promoters were provided during the research.

3.1.3 Measuring weight

To analyse which dietary method stimulate the digestive system and increase the weight, chickens from T1, T2,T3 and T4 were measured. To measure the body weight, from each group five chickens were randomely chosen, the data from each group is given in the table-3. Every week this procedure was followed. During last week, when they were given finisher feed with prospective growth promoters and additives, the T2 group had shown a very good result compared to T1, T3 and T4 group

Table 3: Average body weight of the chickens(5 chickens) from 4 different group were measured in table below

Chicken's group	1st week	2nd week	3rd week	4th week/finale week
T1 (Fed with antibiotics and vitamin)	~120gm	2.377kg	5.119kg	7.950kg
T2 (Fed with garlic)	120 gm	2.415kg	4.832kg	8.760kg
T3 (Fed with garlic and yogurt)	120 gm	2.415kg	4.930kg	7.750kg
T4 (Control)	120 gm	2.264kg	4.630kg	6.490kg

Figure 9: Ahealthy chicken eating from the feeder.

Figure 10: Few chickens from garlic group (T2).

Table 4: Total number of deaths in 4 weeks:

Chicken's group	Number of death chicken
T1	**10**
T2	**9**
T3	**12**
T4	**0**

3.1.4 Health issues found in the antibiotic group: This type of physical abnormality was found in 3 chickens from T1 groups. According to the workers at the farm, this is very common phenomenon. In this study there was no such occurrence in the other groups (T2, T3 and T4).

Figure 11: These chickens were collected from T1 group, these chickens were 22 days old but didn't grow much for their disabilities and for the lack of nutrition they did not survive up to 30days.

3.1.5 Chicken stool samples: Chicken stool samples collected in the falcon tubes with saline water.

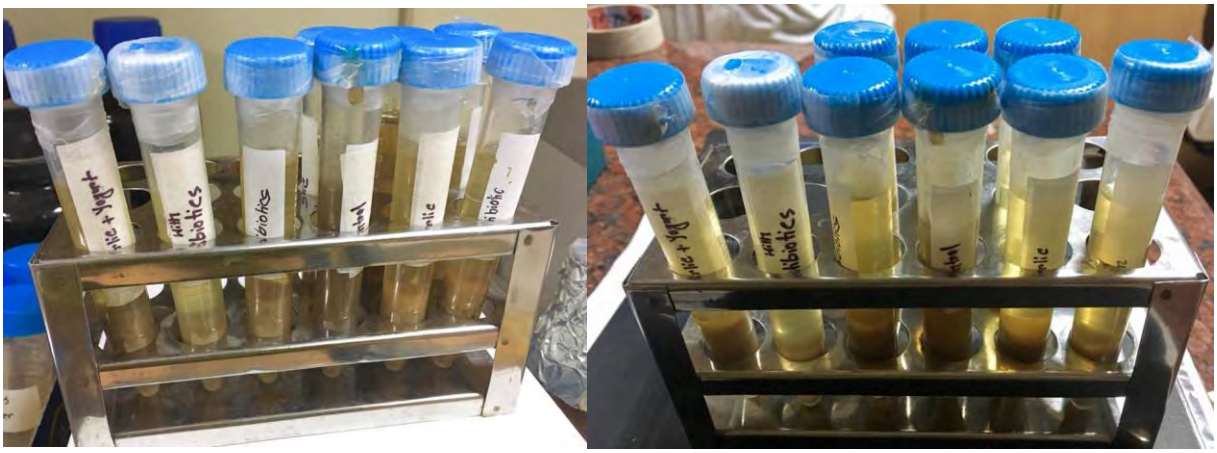
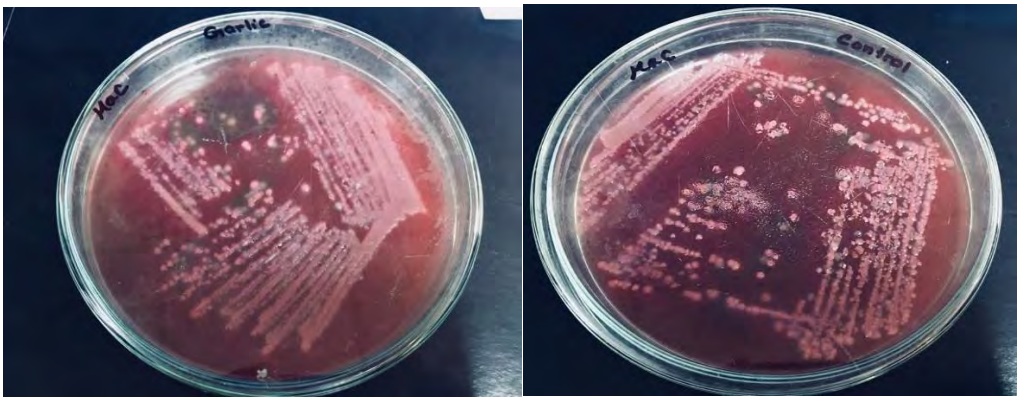


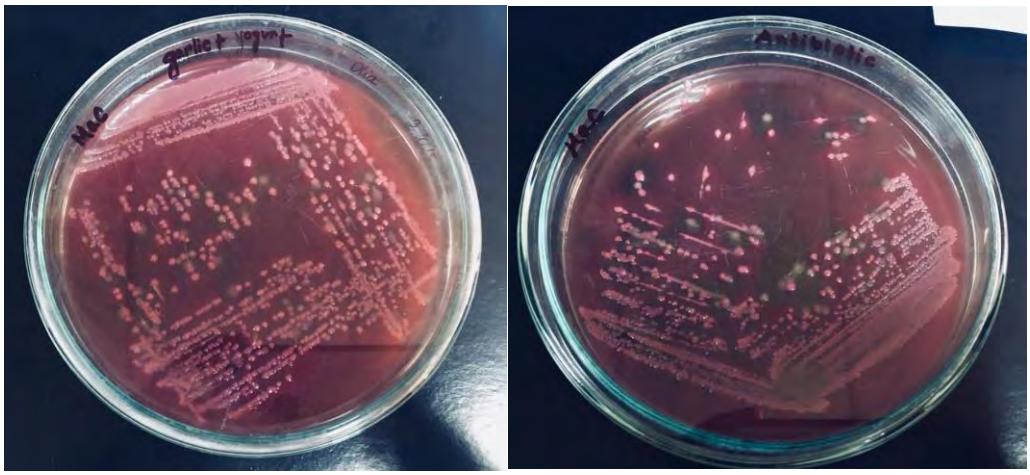
Figure 12: Chicken stool samples which were brought to the laboratory from poultry farm.

3.2 Bacterial growth media: When the samples were brought to laboratory, the feces samples were processed by homogenizing 2-3 grams of each sample in 9ml of sterile physiological saline and then cultured in four types of agar media such as Nutrient agar (NA), MacConkey agar (Mac), eosin methylene blue agar (EMB), salmonella shigella agar (SSA).

3.2.1 Growth of pathogens on MacConkey agar media:



(a) Sample from T2 group. **(b) Sample collected from T4 group**.



(c) Sample collected from T3 group. (d) Sample collected from T1 group.

Figure 13: Sample a,b,c and d were collected from four different groups of chicken, these are chicken feces sample which were brought to the laboratory and grown in a MacConkey agar media.

3.2.2 Growth of pathogen on Nutrient agar media

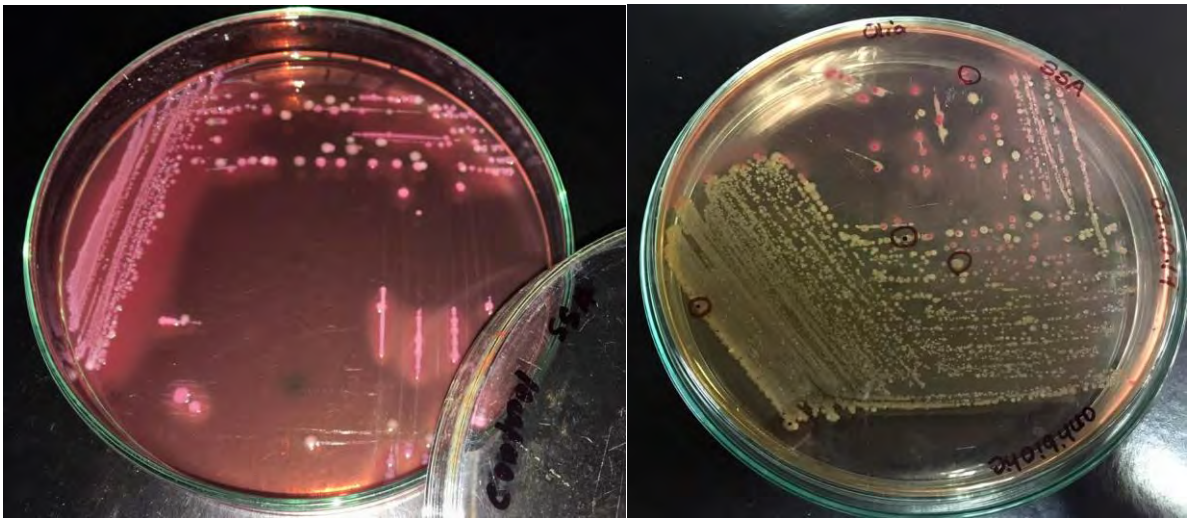
(a) Sample from T2 group. **(b) Sample collected from T3 group.**

(c) Sample collected from T1 group. **(d) Sample collected from T4 group**.

Figure 14: Sample a, b, c, d were collected from four different groups of chicken, these are chicken stools sample which were brought to the laboratory and grown in a Nutrient agar media. White and transparent colonies were identified and then isolated colonies were sub-cultured on nutrient agar media again.

3.2.3 Growth of pathogen on salmonella shigella agar (SSA) media:

(a) Sample from T2 group. **(b) Sample from T3group.**



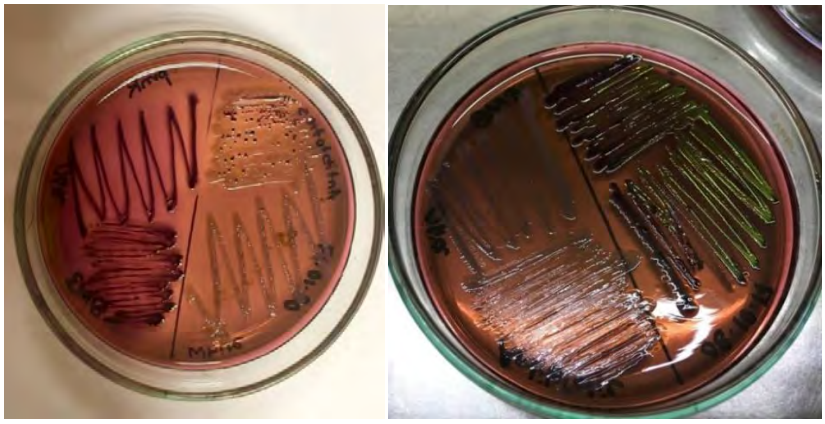
(c) Sample from T4 group. **(d) Sample from T1 group.**

Figure 15: Samples collected from four different groups of chickens were used for streaking on the SSA media. These bacterial growth shows that the samples might contain *Salmonella spp* or *Shigella spp* or both type of organisms.

3.2.4 Growth of pathogen eosin methylene blue (EMB) agar media

(A)(B) (C)

(D) (E) (F)



(G) (H)

Figure 16: A, sample from garlic group (T2), B-sample from garlic and yogurt group (T3), C- sample from antibiotic group (T1), D-sample from control (T4), E- sample from garlic, F sample from control, G- sample from antibiotic group when chickens were 14days old, H-sample from antibiotic group (T1) when the chickens were 22days old.

3.3 Biochemical test results:

3.3.1 Methyl Red test results: The development of a stable red colour in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test.

Figure 17: Samples from all the groups (from left- T2, T3, T4 and T1) showed positive results as they all developed red color in the surface.

3.3.2 Voges–Proskaur test results: In this test, positive test is represented by the development of a red color 15 minutes or more after the addition of the reagents indicating the presence of diacetyl, the oxidation product of acetoin. Here no red colour were formed which indicates tube containing VP negative organisms.

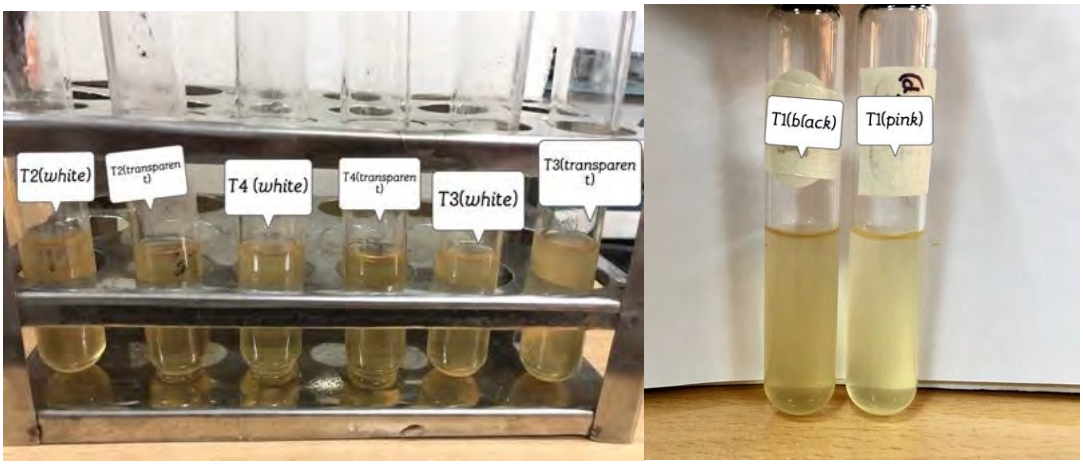


Figure 18: All the test tubes were showed negative results.

3.3.3 Triple Sugar Iron (TSI) test results

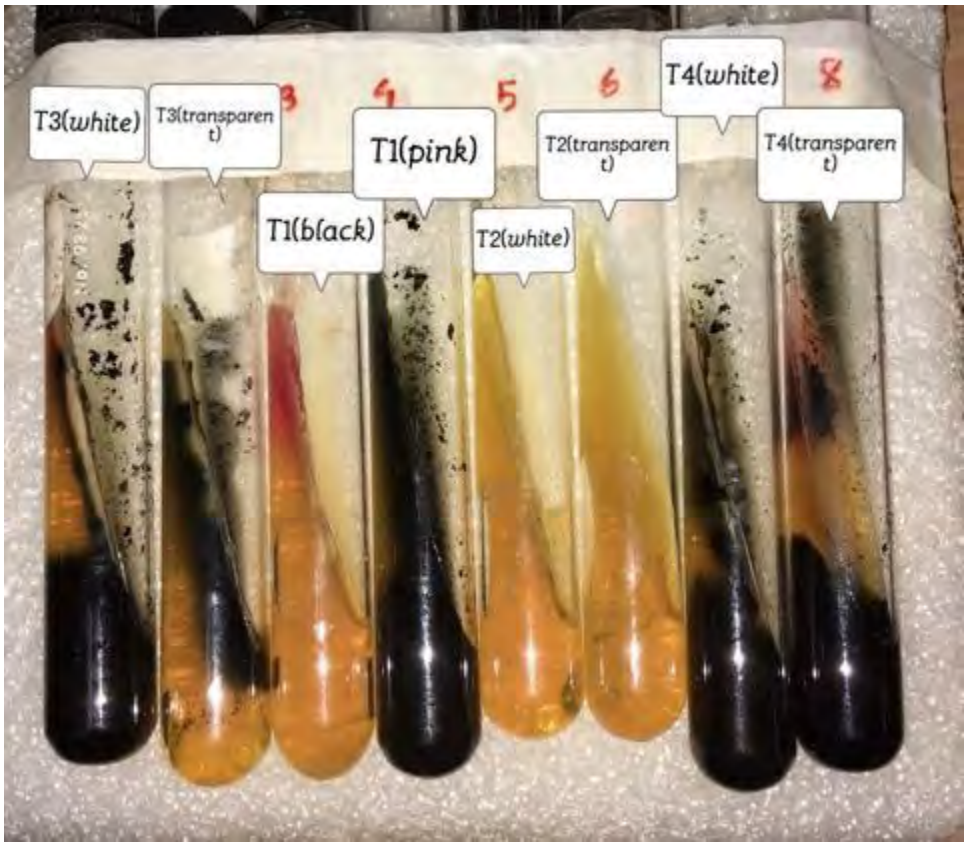


Figure 19: According to the TSI test, yellow slant means acidic condition, red slant means alkaline condition, Black slant means H2S has produced and bubble means gas has produced, here sample from T3 (white) is showing, slant is acidic although little reddish color also appeared, which indicate alkaline condition, H_2S had produced enough to make the butt completely black, no gas had formed where sample from T3 (transparent isolate) is showing black slant in acidic condition, gas had produced that appeared as bubble. Sample from T1 (black isolate) group is showing, alkaline slant and acidic butt, also gas had produced, sample from T1 (pink isolate) group is showing that H2S was produced which turned the whole slant into black. Sample from T2 (white and transparent) group showed no H2S, but gas had produced to make bubbles, both slant turned into yellow (slant and butt) which indicate the acidic condition. Sample from T4 (white isolate) group showed that huge amount of H_2S had produced, no gas or bubble had formed, and acidic or alkaline condition can hardly be interpreted as well as T4 (transparent isolate) group showed huge amount of black slant as a result of H_2S production, little amount of reddish and yellow slant also appeared at top of the tube.

3.3.4 Citrate test results:

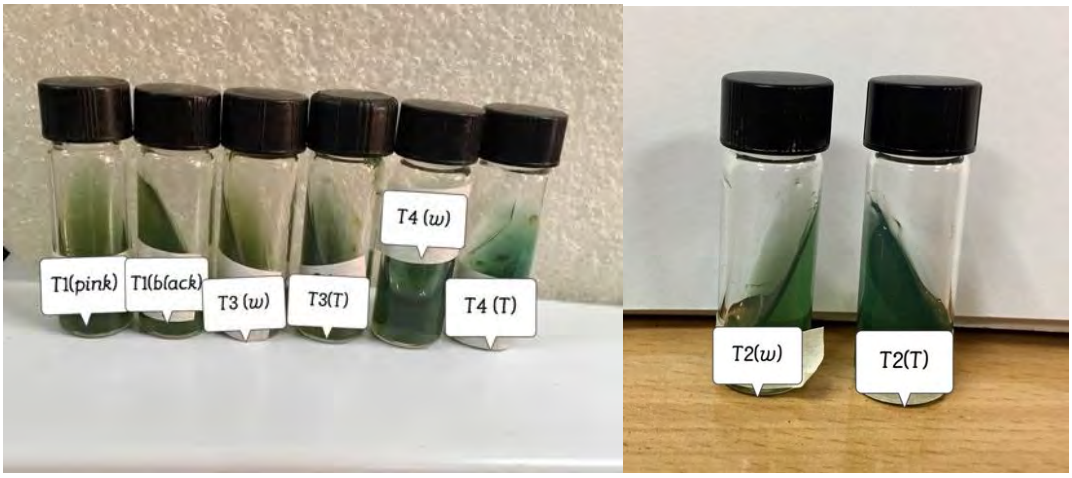
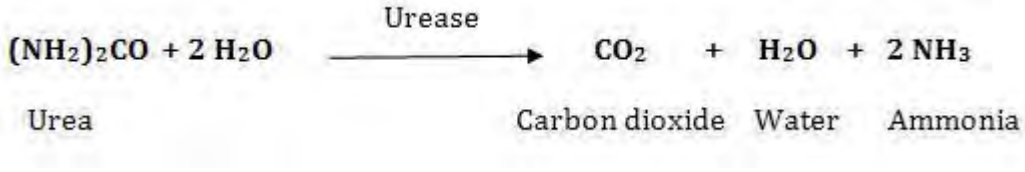


Figure 20: In this test, isolates from all the culture media from T1, T2, T3 and T3 groups showed negative results.

3.3.5 Indole test results:

Figure 21: Positive result were shown by the presence of red colour on the surface alcohol layer of the broth, negative result appeared as yellow color ring on the surface. Here T1 and T4 group showed negative result. Group T3 had one positive result from white isolate and one negative result from transparent isolate. Group T2 showed positive results from both white and transparent isolates.

3.3.6 Urease (MIU) test results: Urease broth is a differential medium that tests the ability of an organism to produce urease. It is hydrolyzed with the release of ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink. If organism does not produce urease the agar slant and butt remain light orange.



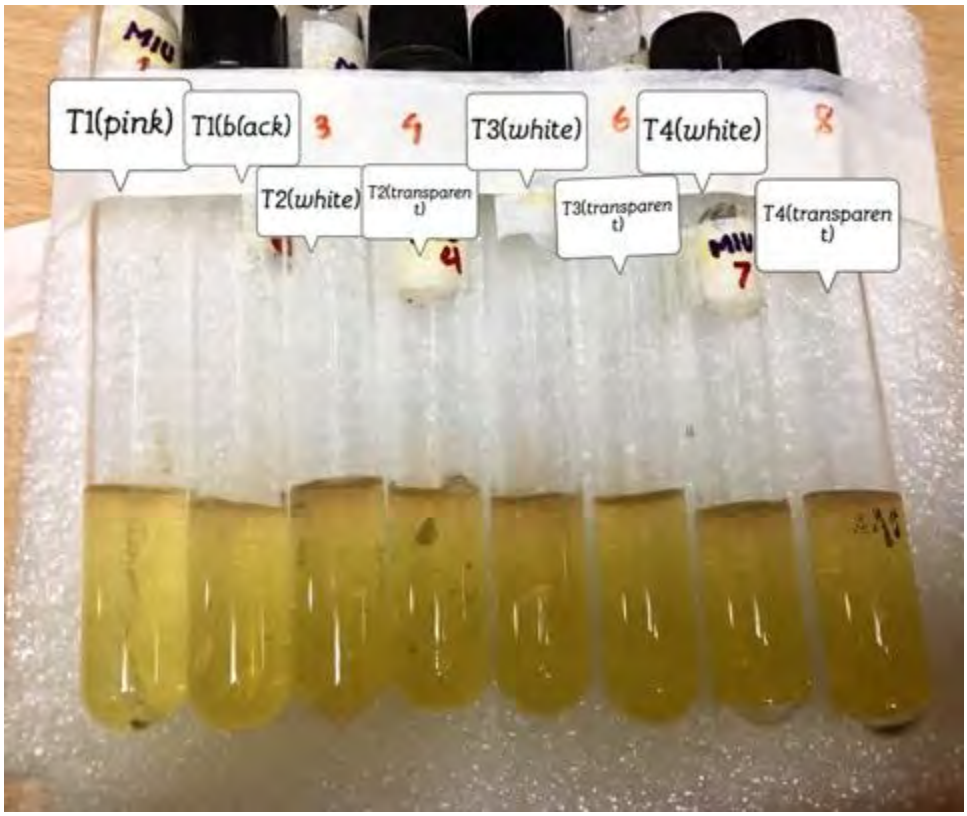
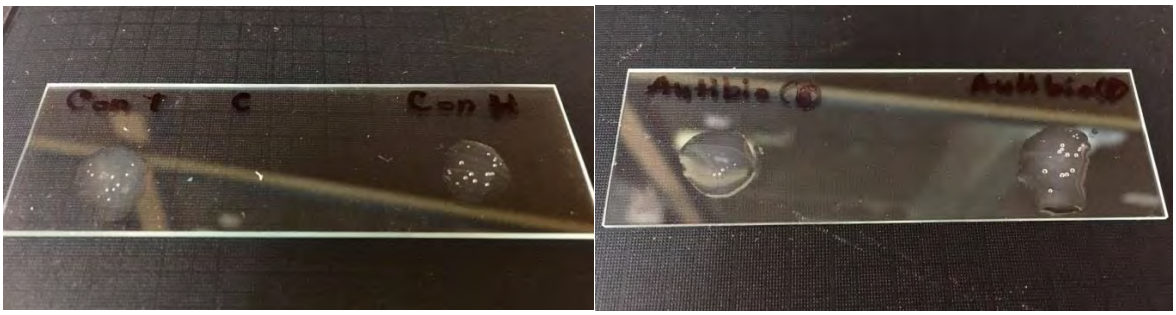


Figure 22: Here organisms in the samples did not produce urease enzyme, so the agar slant and butt remained light orange, which means the results are negative for each group.

3.3.7 Catalase test result: Catalase tests were used as an aid to the identification of Enterobacteriaceae. Members of Enterobacteriaceae family are catalase positive**.** Two main principle for these tests are:

1. Catalase Positive reactions: Evident by immediate effervescence (bubble formation)
2. Catalase Negative reaction: No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide)

Sample from T2 group **sample from T3 group**



Sample from T4 group **sample from T1 group**

Figure 23: All groups gave positive results for catalase test, as bubble was formed.

3.4 Bacterial identifications: Bacteria were identified based on the biochemical test results.

Table 5: Data analysis using online software for biochemical tests:

Chicken Group	Voges Proskaur test	Methyl Red test	Citrate test	Indole test	Urea hydrolysis test	Catalase test	TSI test	Micro-organisms
T2 (White colony)	negative	positive	negative	positive	negative	positive	Yellow slant-yellow butt – glucose, lactose and sucrose fermentation	*E.coli : 99.06%*
T2 (Transparent colony)	negative	positive	negative	positive	negative	positive	Yellow slant and butt glucose, lactose and sucrose fermentation	*E.coli : 99.06%*
T3 (white colony)	negative	positive	negative	positive	negative	Positive	H_2S gas formed but no bubble. Black butt and yellow and little red slant	*E.coli* : 93.07%
T3 (transparent colony)	negative	positive	negative	negative	negative	positive	H2S gas and bubble formed at the end , butt yellow	*Proteus vulgaris : 86.69%* *E.coli : 12.63%*
T1 (pink isolated colony)	negative	positive	negative	negative	negative	positive	H_2S present also whole tube turned into black. No gas formed	*Salmonella typhi : 97.57%* *Salmonella choleraesuis : 1.89%*
T1 (Black isolated colony)	negative	positive	negative	negative	negative	positive	No gas, alkaline slant, acidic butt, no H_2S	*Salmonella paratyphi A : 70.31%* *Salmonella choleraesuis : 28.11%*
T4 (white colony)	negative	positive	negative	negative	negative	positive	H_2S present, no bubble, lactose or sucrose fermentation result unknown	*Salmonella typhi : 97.57%* *Salmonella choleraesuis : 1.89%*
T4 (Transparent colony)	negative	positive	negative	negative	negative	positive	Alkaline slant, no gas or bubble, H_2S present.	*Salmonella typhi : 92.73%*

3.5 Antibiogram test results

All the eight bacterial strains were subjected to the standard disc diffusion test with fourteen antibiotic discs (Table 2). Muller Hinton Agar (MHA) culture media was used for this test. The zone diameter of inhibition interprets the resistance and sensitivity of the organisms to the respective antibiotics. Presence of zone of inhibition around disc means the antibacterial activity against the pathogens

I. For group T1 (black colony):

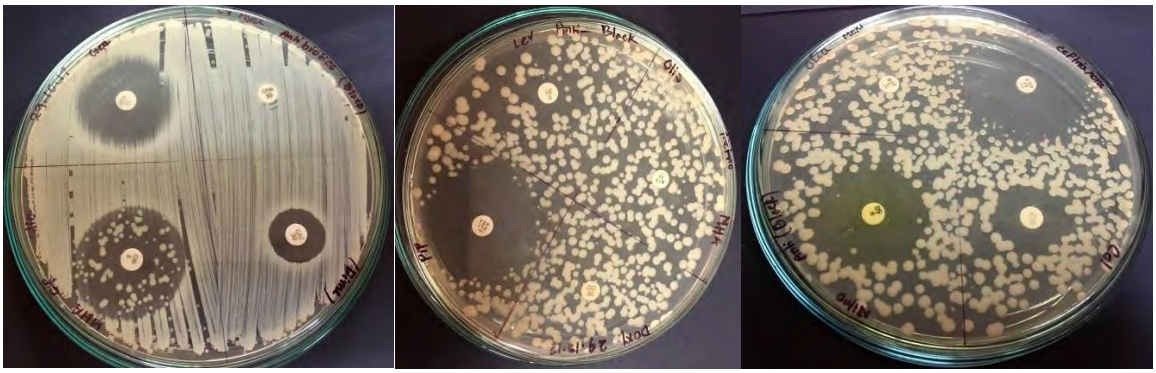


Figure 24: Antimicrobial susceptibility test against the samples collected from T1 group (isolated from black colony) from left – 1.antimicrobial activityofciprofloxacin, Gentamicin, cefeprime, ceftazidime. 2. Levofloxacin, piperacillin-tazobactam, doxycycline, metronidazole. 3. Moxyfloxacin, colistin, nitofurantoin. Ceftriaxone.

II. For group T1 (black colony) and T1 (pink colony):

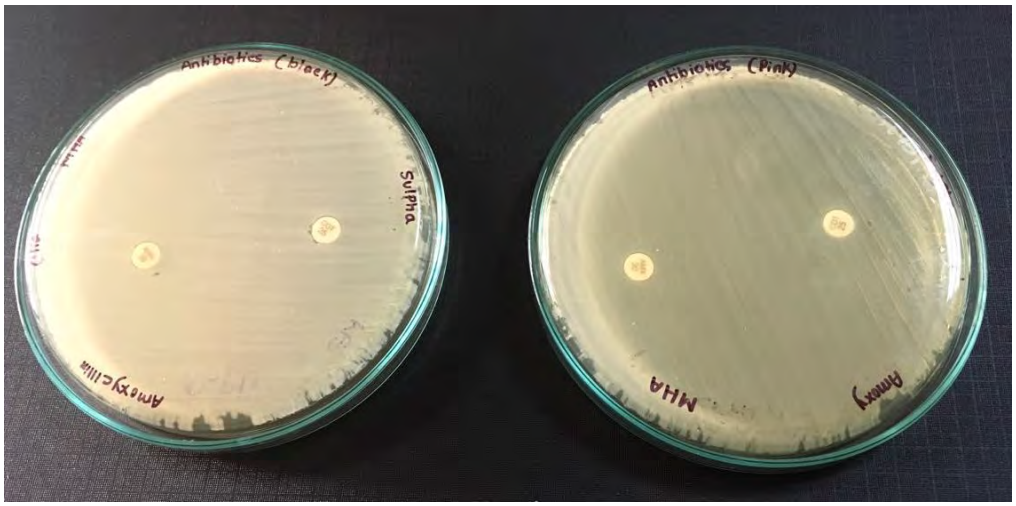


Figure 25: Antimicrobial activity of amoxicillin and sulfamethoxazole on T1 group (chicken) samples of black isolates and pink isolates organisms (from left to right).

III. For Group T1 (pink colony):

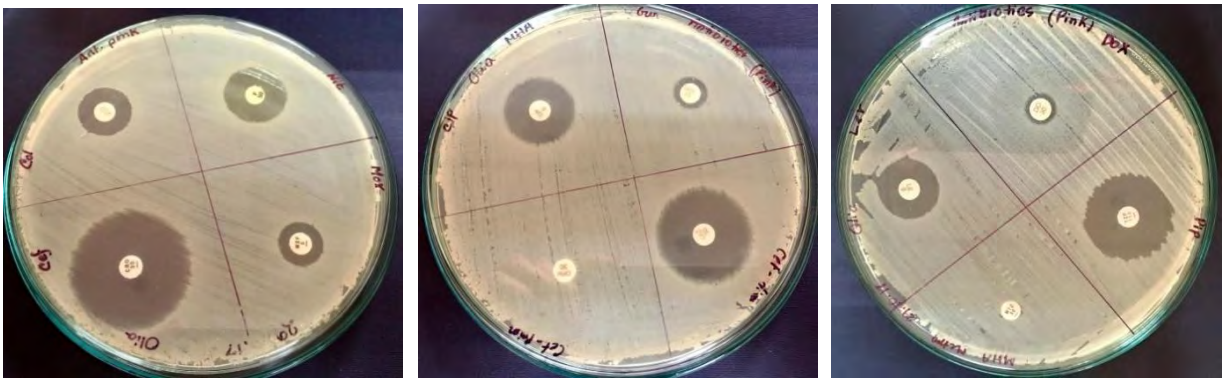


Figure 26: Antimicrobial susceptibility test against the samples collected from T1 group, isolated pink colony, for this test- from the left 1.Moxyfloxacin, colistin, nitofurantoin, ceftriaxone. 2. Ciprofloxacin, Gentamicin, cefeprime, ceftazidime. 3. Levofloxacin, piperacillin-tazobactam, doxycycline, metronidazole.

IV. For group T4 (white colony):

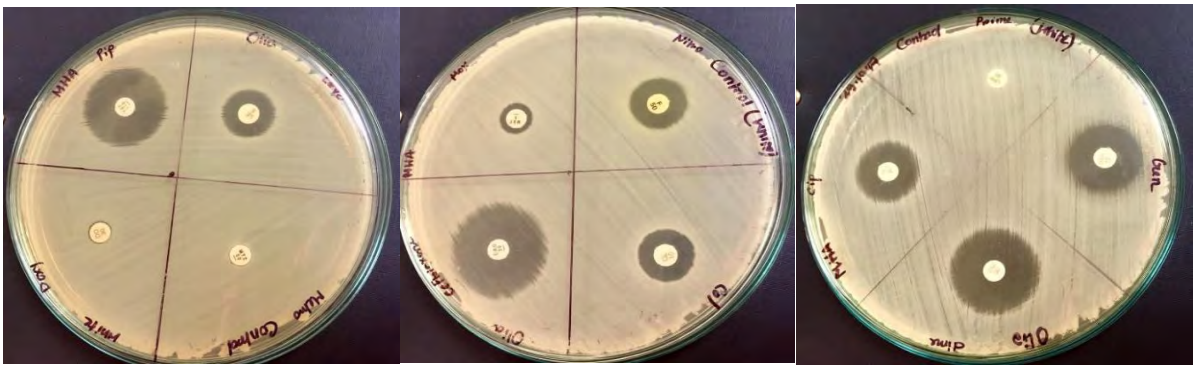


Figure 27: antibiotic disc diffusion against bacteria isolated from control group.From left 1.Levofloxacin, piperacillin-tazobactam, doxycycline, and metronidazole. 2. Moxyfloxacin, colistin, nitofurantoin, ceftriaxone. 3. Ciprofloxacin, gentamicin, cefeprime and ceftazidime.

V. For group T4 (transparent colony):

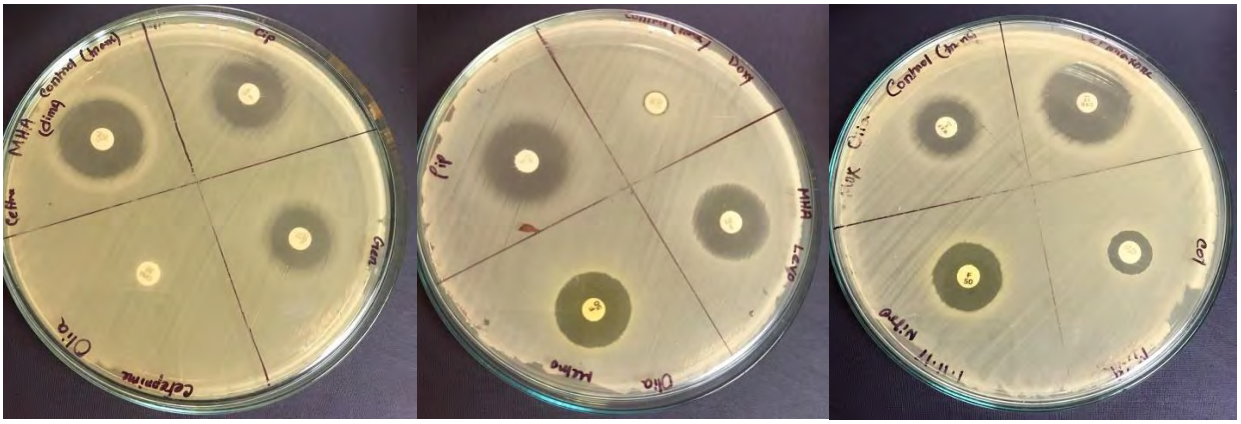


Figure 28: Antimicrobial susceptibility test against collected samples of T4 group. From left- 1.ciprofloxacin, gentamicin, cefeprime, ceftazidime. 2. Levofloxacin, doxycycline, piperacillin-tazobactam, metronidazole. 3.Moxyfloxacin, colistin, nitofurantoin, ceftriaxone.

VI.For group T4 (white colony) and T4 (transparent colony):

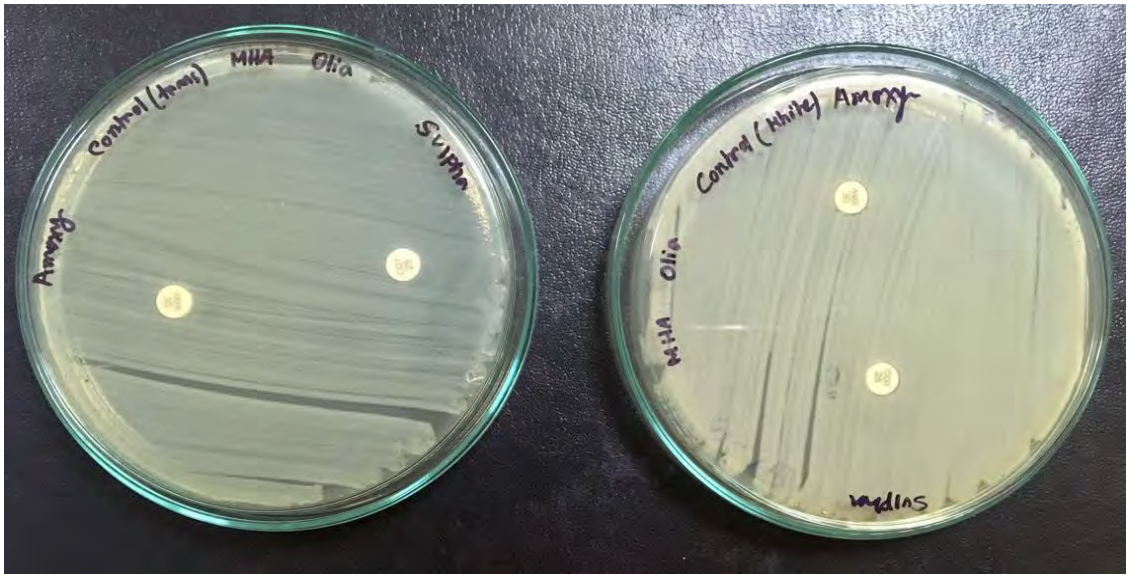


Figure 29: Antimicrobial susceptibility test against collected sample from T4 (transparent colony) group and T4 (white colony) group. From right: sulfamethoxazole and amoxicillin.

VII. For group T2 (white colony):

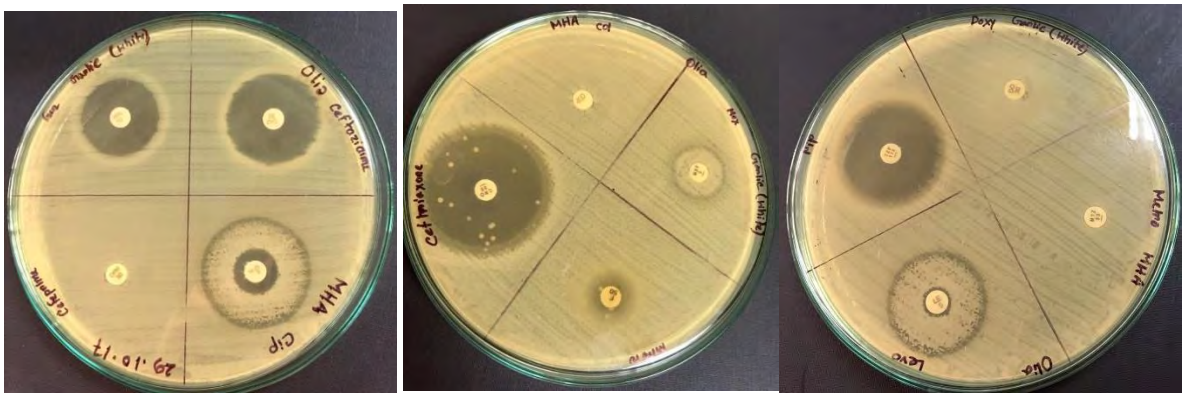


Figure 30: Antimicrobial susceptibility test against collected sample from T2 (white) group. From left- 1. Ciprofloxacin, gentamicin, cefeprime, ceftazidime. 2. Moxyfloxacin, colistin, nitofurantoin, ceftriaxone. 3. Levofloxacin, doxycycline, piperacillin-tazobactam, metronidazole.

VIII.For group T2 (transparent colony):

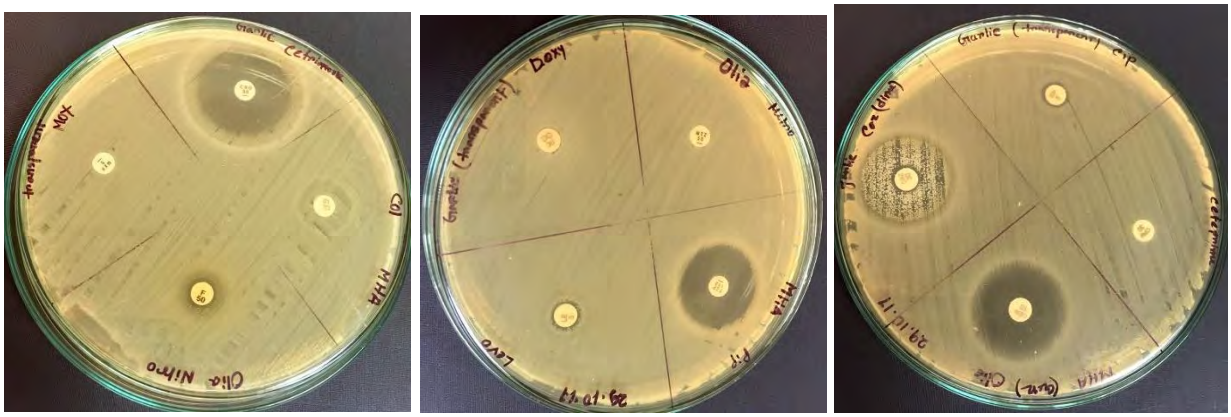


Figure 31: Antimicrobial susceptibility test against collected sample from T2 (transparent colony) group. From left-1. Moxyfloxacin, colistin, nitofurantoin, ceftriaxone. 2. Levofloacin, doxycycline, piperacillin-tazobactam, metronidazole. 3. Ciprofloxacin, gentamicin, cefeprime, ceftazidime.

IX. For group T2 (white colony) and T2 (transparent colony) :

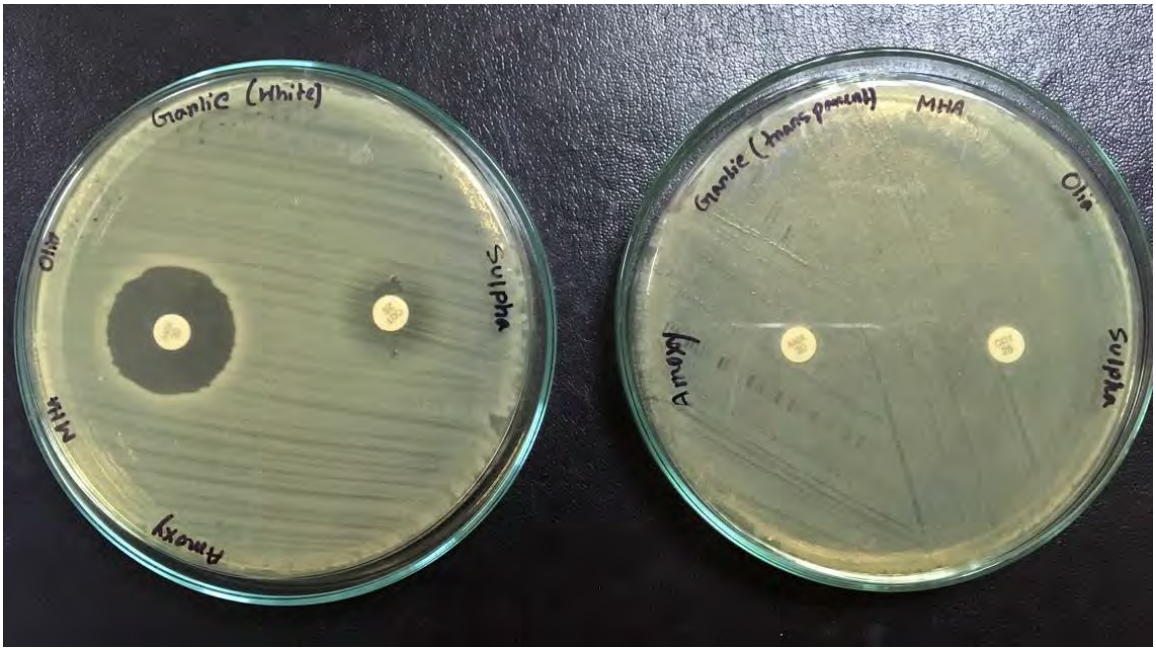


Figure 32: Antimicrobial susceptibility test against the white and transparent colored isolates from T2 group, from left: amoxicillin and sulfamethoxazole.

X.For group T3 (white colony) group:

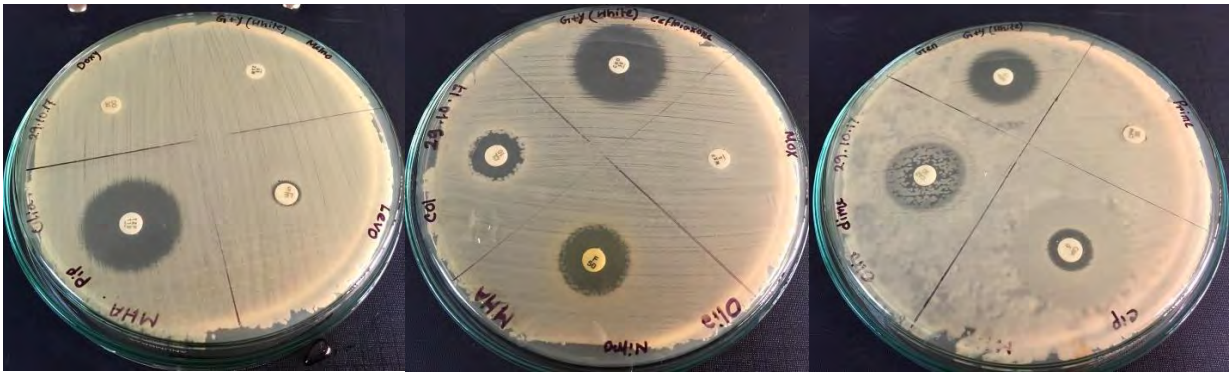


Figure 33: Antimicrobial susceptibility test against the sample from T3 (white colony). From left- 1 Levofloxacin, doxycycline, piperacillin-tazobactam, and metronidazole. 2. Moxyfloxacin, colistin, nitofurantoin, ceftriaxone. 3. Ciprofloxacin, gentamicin, cefeprime, ceftazidime.

XI. For group T3 (transparent colony):

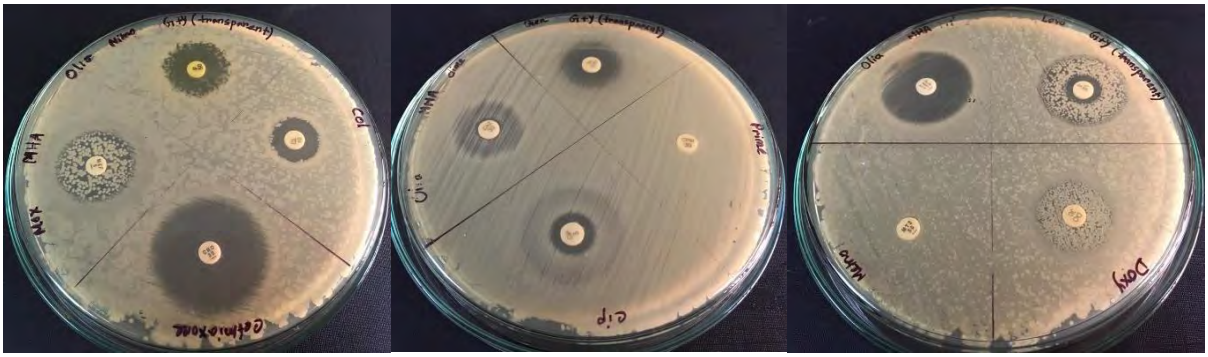


Figure 34: From left- 1. Moxyfloxacin, colistin, nitofurantoin, ceftriaxone 2. Ciprofloxacin, gentamicin, cefeprime, ceftazidime. 3. Levofloxacin, doxycycline, piperacillin-tazobactam and metronidazole.

XII. For group T3 (white colony) and T3(transparent colony) group:

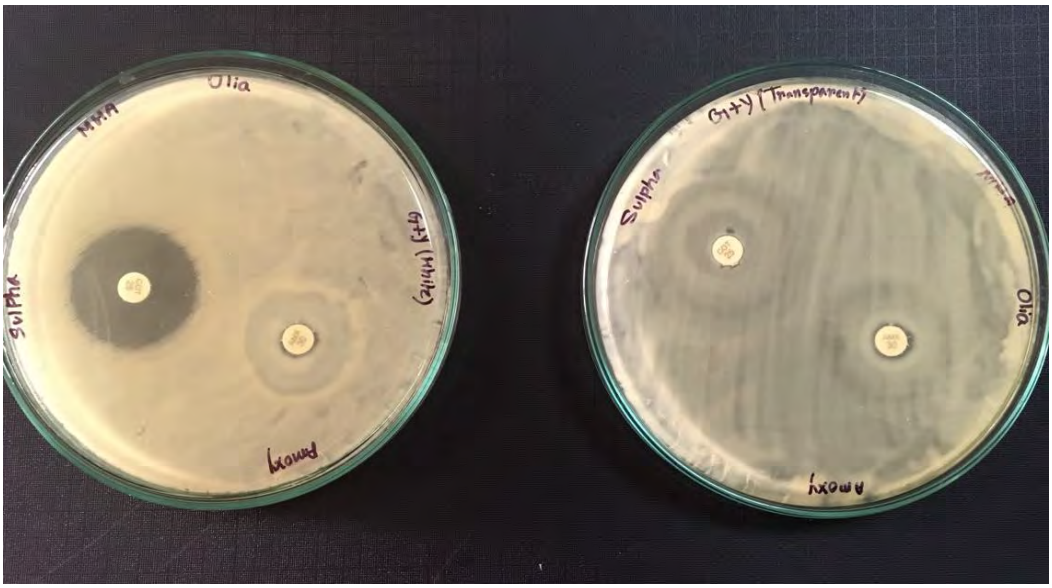


Figure 35: Antibiotic susceptibility test against the T3 group, from left: sulfamethoxazole and amoxicillin.

3.5.1 Interpretation of Antibiotic susceptibility test

Table 6: Antibiotic susceptibility test against isolated pathogen from chicken's stool samples collected from T1 group

Antibiotic's name (30 ug)	Zone of Inhibition in mm			
	T1/ black colony	Interpretation	T1/ pink colony	Interpretation
Amoxicillin	-	Resistant	0	Resistant
Ceftazidime.	16	Intermediate	26	Sensitive
Ceftriaxone	39	Sensitive	32	Sensitive
Cefeprime	-	Resistant	0	Resistant
Ciprofloxacin	12	Resistant	20	Intermediate
Colistin	18	Sensitive	12	Sensitive
Doxycycline	-	Resistant	9	Resistant
Gentamicin	30	Sensitive	11	Resistant
Levofloxacin.	-	Resistant	19	Sensitive
Piperacillin/ Tazobactam	37	Sensitive	25	Sensitive
Metronidazole.	-	Resistant	0	Resistant
Moxifloxacin.	-	Resistant	13	Resistant
Nitrofurantoin.	31	Sensitive	20	Sensitive
Sulfamethoxazole.	-	Resistant	0	Resistant

Table 7: Antibiotic susceptibility test against isolated pathogen from chicken's stool samples collected from T2 group

Antibiotic's name (30mg)	Zone of Inhibition in mm			
	T2/white colony	Interpretation	T2/transparent colony	Interpretation
Amoxicillin	26	Sensitive	-	Resistant
Ceftazidime.	27	Sensitive	7	Resistant
Ceftriaxone	20	Intermediate	30	Sensitive
Cefeprime	-	Resistant	0	Resistant
Ciprofloxacin	14	Resistant	9	Resistant
Colistin	-	Resistant	0	Resistant
Doxycycline	-	Resistant	0	Resistant
Gentamicin	25	Sensitive	24	Sensitive
Levofloxacin.	6	Resistant	0	Resistant
Piperacillin/ Tazobactam	30	Sensitive	25	Sensitive
Metronidazole.	-	Resistant	0	Resistant
Moxifloxacin.	-	Resistant	0	Resistant
Nitrofurantoin.	-	Resistant	0	Resistant
Sulfamethoxazole.	-	Resistant	0	Resistant

Table 8: Antibiotic susceptibility test against isolated pathogen from chicken's stool samples collected from T3 group

Antibiotic's name (30ug)	Zone of Inhibition in mm			
	T3/ white colony	Interpretation	T3/ Transparent colony	Interpretation
Amoxicillin	-	Resistant	-	Resistant
Ceftazidime.	-	Resistant	-	Resistant
Ceftriaxone	29	Sensitive	32	Sensitive
Cefeprime	-	Resistant	-	Resistant
Ciprofloxacin	11	Resistant	16	Intermediate
Colistin	14	Sensitive	15	Sensitive
Doxycycline	-	Resistant	-	Resistant
Gentamicin	22	Sensitive	19	Sensitive
Levofloxacin.	8	Resistant	14	Intermediate
Piperacillin/ Tazobactam	26	Sensitive	29	Sensitive
Metronidazole.	-	Resistant	-	Resistant
Moxifloxacin.	-	Resistant	-	Resistant
Nitrofurantoin.	18	Sensitive	20	Sensitive
Sulfamethoxazole.	30	Sensitive	-	Resistant

Table 9: Antibiotic susceptibilitytest against isolated pathogen from chicken's stool samples collected from T4 group

Antibiotic's Name (30 ug)	Zone of Inhibition in mm			
	T4/ white colony	Interpretation	T4/ Transparent colony	Interpretation
Amoxicillin	-	Resistant	-	Resistant
Ceftazidime.	25	Sensitive	23	Sensitive
Ceftriaxone	28	Sensitive	26	Sensitive
Cefeprime	-	Resistant	-	Resistant
Ciprofloxacin	20	Intermediate	20	Intermediate
Colistin	16	Sensitive	14	Sensitive
Doxycycline	-	Resistant	6	Resistant
Gentamicin	21	Sensitive	17	Sensitive
Levofloxacin	16	Intermediate	21	Sensitive
Piperacillin/ Tazobactam	25	Sensitive	22	Sensitive
Metronidazole	-	Resistant	20	Sensitive
Moxifloxacin.	10	Resistant	16	Intermediate
Nitrofurantoin.	16	Intermediate	20	Sensitive
Sulfamethoxazole.	-	Resistant	-	Resistant

Table 10: The table below is made based on the Interpretation of antibiogram test results of each group for comparison purpose.

Resistant = R, Intermediate = I and Sensitive = S

Antibiotic's name	T1/ *Salmonella*	T1/ *Salmonella*	T2/ *E.coli*	T2/ *E.coli*	T3/ *E.coli*	T3/ *Proteus vulgaris*	T4/ *Salmonella*	T4/ *Salmonella*
Amoxicillin	R	R	R	R	R	R	R	R
Ceftazidime	I	S	R	R	R	R	S	S
Ceftriaxone	S	S	S	S	S	S	S	S
Cefeprime	R	R	R	R	R	R	R	R
Ciprofloxacin	R	I	R	I	R	I	I	I
Colistin	S	S	S	S	S	S	S	S
Doxycycline	R	R	R	R	R	R	R	R
Gentamicin	S	R	S	S	S	S	S	S
Levofloxacin	R	S	R	I	R	I	I	S
Piperacillin-Tazobactam	S	S	S	S	S	S	S	S
Metronidazole	R	R	R	R	R	R	R	S
Moxifloxacin	R	R	R	R	R	R	R	I
Nitrofurantoin	S	S	S	S	S	S	I	S
Sulfamethoxazole	R	R	S	R	S	R	R	R

- **Here it is shown that one isolate form T1 group was resistant to 8 antibiotics and another isolate from the same group was resistant against 7antibiotics. Similar results were obtained in case of T2 and T3 groups.**
- **One isolate form T4 (Control) group was resistant to 6 antibiotics and another isolate from the same group was resistant against 4 antibiotics.**

Here, except T1 group, all groups were sensitive against gentamicin. It is quite frightening, that T1, T2 and T3 group showed resistance against ciprofloxacin and moxifloxacin. All the samples from each group showed resistance to doxycycline, cefeprime and amoxicillin. Isolates from T1 and T4 groups showed resistance to sulfamethoxazole. All the samples were showed sensitivity to cefriaxone, colistin, piperacillin-tazobactam and nitrofurantoin.

Chapter.04
Discussion and conclusion

Discussion:

In the poultry farm, bedding is most important to keepthe chickens warm, especially when they are first broughtinto the poultry house.Broiler litterwhich is a mixture of poultry excreta, spilled feed, feathers and material (wooden and rice brans) is used as bedding.Chicken litter is one of the main sources of bacterial contamination in the broiler houses. In order to comply with the proper maintenance rules, the chicken litters need to be changed regularly as hundreds of chickens live in a particular premise especially since there will still remain a few contaminants which can contaminate the chickens, their feed or water. Like any other poultry houses, in this farmit was a very common scenario which was very responsible for bacterial infections. Usuallychicken's intestines is the most infected organ where Enterobacteriaceae is known to survive and create common diseases for chickens.Several pathogenic Enterobacteriaceae, specially *Escherichia coli* strains, cause diarrhoea, urinary tract infections, mastitis, arthritis and meningitis (Martin et al., 2014). Although many of these pathogenic bacteria recovered from poultry have been monitored, several published studies have reported on antimicrobial resistance in pathogenic bacteria found in poultry, particularly *Salmonella* and *Escherichia coli* (Chung et al., 2004). In this research study, when all the biochemical tests were done in the laboratory, the isolated organisms were *E.coli*, *Proteus vulgaris*, *Salmonella typhi, Salmonella choleraesuis, Salmonella paratyphi* A. and similarly they also showed resistance against multiple antibacterial drugs.

In this study, group T1 chickens were fed antibiotics as a growth promoter and also other artificial supplements such as vitamins, prescribed by the veterinarian. It is a very regular and common case for any poultry farms. But for this study, the main focus was to find alternative way to treat chickens. The group T2 and T3, were made to find that alternative rather than following the prescribed drugs. Group T2 chickens were fed garlic powder with water from 2 days old but when the chickens got older, according to that the amount of garlic got higher. This was done so that the chickens could easily adjust to their internal digestive system with the increasing amount of doses. At final week, which is basically after 21days, the total body weights of this group of chickens showed higher value as compared to other groups (table 3) which proves the fact that garlic as a natural growth promoter can be a potential alternative to common artificial growth promoters like antibiotics. (Demir et at., 2003). A study was conducted in 2016, where it showed similar results, chickens given garlic as a supplementary diets showed significantly ($p<0.05$) higher values as

compared to control and combination of garlic and ginger supplemented group(Karangiya et al., 2016).

Also the numbers of deaths of chickens were also moderate and lesser in T2 group. No physical abnormalities or diseases like diarrhea were found. Another important finding that was observed after garlic water consumption is that less amount of bad odor in the air was detected. Ammonia is a gas present in the atmosphere of every poultry house and causes bad odors. At high concentration it irritates the mucous membranes of the respiratory system, increases the susceptibility of chicken to bacterial respiratory infection, especially *E. coli* infection. High levels of ammonia also have a negative impact on the overall livability, weight gain, feed conversion and condemnation rate at processing and the immune system of the chickens. Nuisance and odor control continue to be major challenges to livestock and poultry producer (JR, 1997). An extensive report on the odors from broiler farms in Australia by the Rural Industries Research and Development Committee has shown that aerobic microbial metabolism processes in the litter produce nitrogen-containing odorants such as ammonia, amines, indole, skatol, and volatile fatty acids as well as sulfur-containing odorants such as hydrogen sulfide, dimethyl disulfide, and dimethyl trisulfide (Enticknap et al., 2006) In terms of antibiotic potency against Gram-negative bacteria of garlic, it can cancel the sulfhydryl compounds when it is provided in aquatic diet (Fujisawa et al., 2009). Therefore, garlic can eliminate bad odors by cancelling sulfur containing compounds. The garlic group chickens were found to be healthiest and the workers, who were responsible for taking care of the poultry farm, confirmed that there was lesser amount of bad odor created by this group. According to Glenn Birrenkott, Clemson animal and veterinary science professor, including garlic powder in the proportion of 3% to their total feed makes the poultry house like a pizzeria instead of manure. Now after this experiment it was confirmed that Dr Glenn was not wrong. It affects the body weight and digestion systems in a good way and is proven to be very beneficial for health as well. During the process of collecting stool samples from this group, no runny or watery droppings from chicken were found, this may indicate a healthy digestive systems of this particular chickens group. After running biochemical tests of chicken feces sample, this group confirmed that there were no *Salmonella*, although the presence of *E.coli* was confirmed. The percentage of deaths of chickens was also lesser than T1, T3 groups. So it can be concluded that garlic supplementation improved the overall performance and health of broilers.

Similarly chickens from garlic and yogurt group (T3) were observed by measuring their weights. Now there were few drawbacks in this group, such as during collecting samples few chickens were observed to give watery droppings, which might be a result of intestinal infections. This group was given garlic water solely up to 13 days age. At day 14 they were first introduced with yogurt. In this research, they were given yogurt (without sugar) from Aarong Dairy because of the probiotic activity. The reason why this group of chickens had suffered from diarrheal symptoms might be because of the failure of measuring a standard amount of yogurt feed for chickens. Maybe a standard dose could have reduced this consequence. As this study was conducted for research purpose, an experimental dietary was followed to see the result. After the biochemical tests, it was confirmed that feces sample from this group of chickens were highly contaminated with *Proteus vulgaris, E.coli* (table 5). This group had slightly fallen in the scale of body weights. On the contrary, the atmosphere had lesser amount of ammonia gas odor than the T1 and T4 groups. The average body weight of the chickens from this group was higher than control (T4) group but lower than T1 and T2 group. Maybe a standard amount or more reliable source of yogurt supplement could have improved the health condition of this group.

Although this group has drawbacks but there are few positive outcomes also noted down, such as the costs of yogurt and garlic (natural supplements) are much lower than antibiotics and chemically synthesized hormones. The production of bad odors was also very moderate and tolerable as compared to T1 and T4 group.

Correspondingly the T4 group had its own pros and cons. As this group was not provided with medicines or other beneficial natural substances unlike other groups, this group had only 25 chickens instead of 200. The main reason for choosing this amount of chicken was to reduce the loss due to poor immune systems they were given no medications or growth promoters to fight against disease and infections. Therefore, the chickens could easily die if any deadly predators or entities attacked in their system. Any health hazard could have easily led to death. The money is an important factor too as losing so many chickens could affect the poultry business badly. So for this research study, 25 chickens were selected as a control group. In the end, no chicken was found dead from this group. This is a very interesting finding. There are a few reasons that might be responsible for this: (1) few number of chickens means more space for proper ventilation. (2) No

chemical or strong substances were provided. (3) These chickens did not have to adjust their digestive system to any supplements or additives. (4) A decent space to move freely. (5) They had dealt with less amount of ammonia, uric acid or other toxic substances released from the chickens. The total cost for raising chickens of T4 group was lesser than the other groups andno physical disability was found in this group. However, the total body weights of the control group chickens had dropped compared to the other groups. When the stool samples from this group were analyzed at the laboratory, family of Enterobacteriaceae such as *Salmonella typhi* and *Salmonella choleraesuis* were found.

Similarly like other groups, T1 group was strictly under observation. The total body weight of the chickens from this group was higher than T3 and T4 although it was lower than T2 group. Garlic had shown better results in terms of health matters. The total mortality rate of T1 group was higher than T3 and almost as similar as T2. There are few findings that were observed during the study which was that the bad odor in the air from this group was quite noticeable whereas chickens from T2 and T3 showed very less odors. Later it was stated that total body weight of T1 group chickens did not increase significantly during finale week as compare to T2. As this group was given antibiotics and other food additives (commercially manufactured) so the costs were higher as well. Commercially manufactured antibiotics and other growth promoters cost around 717-/ (seven hundred and seventeen BDT) where garlic cost only 70-/ BDT and yogurt also costs 70-/ BDT.

Antibiotics as growth promoters that were provided to this group (T1) were colistin, amoxicillin sulfamethoxazole and vitamin c, after analyzing the feces samples through biochemical tests, *Salmonella typhi* (97.57%), *Salmonella paratyphi A (70.31%), Salmonella choleraesuis* (28.11%)were found from this group. After performing antibiogram tests, the presence of multidrug resistant pathogens was confirmed in this group (Table 8).Multidrug resistant pathogens were found in each group. Although ciprofloxacin rarely used in poultry farming (Adelaide, 2008), all the groups showed resistance against it. Ciprofloxacin is a broad-spectrum antibiotic widely prescribed in clinical and hospital settings. The emergence of antimicrobial resistance against effective antibiotics is a global issue. A study conducted in Karachi, Pakistan, investigated the present status of antimicrobial resistance against ciprofloxacin, five hundred and twenty four clinical isolates of *Escherichia coli* (30%) and *Salmonella typhi* (9%) and showed that ciprofloxacin is 27.02% and 16.66% resistant to *Escherichia coli* and *Salmonella typhi*

respectively due to its irrational and inappropriate use (Ali et al., 2010) which is quite relatable to this research study. Here feces samples contaminated with pathogens showed antimicrobial resistance. In one of the studies conducted by Wright State University, it concluded that garlic as an antibiotic is actually as potent as penicillin by one percent. Even Louis Pasteur in the 19th century conducted his own experiment and demonstrated how it destroyed bacteria under laboratory conditions. As it is known, the body can develop resistance against antibiotics especially if it is not taken regularly as it should be. This is when garlic becomes advantageous because the human body does not develop resistance against it. In this research, the effect of garlic was not fruitful as it should be as resistant pathogen were isolated from the garlic fed chicken's feces but in terms of body weight, odor and mortality rates, it proves to be a very useful and cost efficient. However, test of more samples for antibiotic resistance could have given different results.

Another finding was noticed from this study that piperacillin-tazobactam showed susceptibility against all the feces samples from T1, T2, T3 and T4. After this study it is proved that piperacillin-tazobactam is a very good source of antibiotic which is a combinational drug and bacteria could not grow against them. A study conducted at National Committee for Clinical Laboratory Standards (USA) proved that the spectrum of activity of piperacillin-tazobactam for empirical antibiotic therapy is significantly greater than that of piperacillin alone and is similar to that of ciprofloxacin and gentamicin (Cormican et al, 1998) which is also quite relatable to this study (Table 8), here colistin, piperacillin-tazobactam, ceftazidime, nitrofurantoin, ciprofloxacin, Gentamicin showed better results compared to other antibiotics.

Another finding was observed during this research, few chickens were found with leg paralysis from T1 group. During the time of third week, suddenly 3 chickens become paralysis and could not move and later they stopped eating and died at the 4th week. Now this outcome was very unusual as no other groups had shown this type of abnormalities in chickens. The reason behind of this abnormality is still unknown. Maybe it could be caused by the artificial chemical substances released from the drugs that had been used for this group.

Conclusion:

The Present study showed an overall positive result in the growth of broiler chickens when garlic was fed. Even in terms of mortality, garlic fed chickens showed a comparatively better results than the antibiotic fed chickens. This indicates garlic as an alternative to antibiotics used in poultry business. In future, it can be expected to be incorporated in the poultry industry as a cheaper and more effective substitute for the expensive synthetic drugs. However, this study should be repeated with a larger sample size to provide more credible and significant results to reveal the correlation of feeding antibiotics with drug resistance.

References

- Alena Klochko (2011) Salmonellosis treatment and management.Medscape.
- O A Adelaide, Christine C. Bii, Paul Owour Okemo (2008 Dec). Antibiotic resistance and virulence factors in Escherichia coli from broiler chicken slaughtered at Tigoni processing plant in Limuru, Kenya. *East African Medical Journal*, 85(12):597-606.
- Arufa Parveen, Md. Mostafizer Rahman, Md. Fakhruzzaman1, Mir Rowshan Akter and Md. Shofiqul Islam (2017). Characterization of bacterial pathogens from egg shell, egg yolk, feed and air samples of poultry houses., 3 (2), 168-174.
- Suzanne D. Caudry and Vilma A. Stanisich(1979). Incidence of antibiotic-resistant Escherichia coli associated with frozen chicken carcasses and characterization of conjugative R plasmids derived from such strains. *Antimicrob Agents Chemother.;* , 701-9.
- Chung YH1, Kwon YI, Kim SY, Kim SH, Lee BK, Chang YH (2004.). Antimicrobial susceptibilities and epidemiological analysis of Salmonella enteritidis isolates in Korea by phage typing and pulsed-field gel electrophoresis. Food Prot. 67:264–270.
- E. Demir, Şenay Sarica, M. A. Özcan and M. Suiçmez(2003),The use of natural feed additives as alternatives for an antibiotic growth promoter in broiler diets,69 (3). S. 110–116
- E. Demir1,Şenay Sarica, M. A. Özcan and M. Suiçmez (2003). The use of natural feed additives as alternative to an antibiotic.69 (3). S. 110–116
- Javandel F1, Navidshad B, Seifdavati J, Pourrahimi GH, Baniyaghoub S (2008). The Favorite Dosage of Garlic Meal as a Feed Additive in Broiler Chickens Ratios. *Pakistan Journal of Biological Sciences,*, 11: 1746-1749.
- Fujisawa H1, Watanabe K, Suma K, Origuchi K, Matsufuji H, Seki T, Ariga T (2009). Antibacterial potential of garlic-derived allicin and its cancellation by sulfhydryl compoundsBiosci Biotechnol Biochem.;73(9):1948-55.
- Hanieh H1, Narabara K, Piao M, Gerile C, Abe A, Kondo Y .(2010). Modulatory effects of two levels of dietary Alliums on immune response and certain immunological variables, following immunization, in White Leghorn chickens.Anim Sci J. 2010 Dec;81(6):673-80.
- Varmaghany S1, Karimi Torshizi MA2, Rahimi S3, Lotfollahian H4, Hassanzadeh M (07 June 2015). The effects of increasing levels of dietary garlic bulb on growth performance, systolic blood pressure, hematology, and ascites syndrome in broiler chickens. Poultry science.1812–1820,
- C. W. Blackburn, Clive de W. Blackburn, Peter McClure, P. J. Mcclure (2002). Salmonella, Foodborne Pathogens: Hazards, Risk Analysis and Controlpage: 521.

- Bauer-Garland J1, Frye JG, Gray JT, Berrang ME, Harrison MA, Fedorka-Cray PJ. (2006). Transmission of Salmonella enterica serotype Typhimurium in poultry with and without antimicrobial selective pressure. *Appl. Microbiol101(6):1301-8.*.
- Miner JR (1997). Nuisance concerns and odor control. 80(10):2667-72.
- Julie J. Enticknap, Hirofumi Nonogaki, Allen R. Place* and Russell T. Hill (2006). Microbial Diversity Associated with Odor Modification for Production of Fertilizers from Chicken Litter.Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202.
- Knarreborg A1, Simon MA, Engberg RM, Jensen BB, Tannock GW. (2002). Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Appl Environ Microbiol*, 68(12):5918-24.
- Cormican M1, Corbett-Feeney G, Kelly S, Hughes D, Flynn J, Jones RN. (1998). In-vitro activity of piperacillin/tazobactam relative to other antibiotics against blood culture isolates. *Irish Journal of Medical Science,*, Volume 167, Number 3, Page 155.
- Martin Mellen1, Miroslava Kačániová2, Jana Petrová2 , Henrieta Arpášová3 , Marcela Capcarová4 , Peter Haščík5 (2014,). Enterobacteriaceae Genera Representation of Chicken's Gastrointestinal Tract after Feed Additives Application . *Animal Science and Biotechnologies,* 47 (2).
- Moussa S. Diarra, Fred G. Silversides, Fatoumata Diarrassouba, Jane Pritchard, Luke Masson, Roland Brousseau, Claudie Bonnet, Pascal Delaquis, Susan Bach, Brent J. Skura,and Edward Topp6 (2007). Impact of Feed Supplementation with Antimicrobial Agents on Growth Performance of Broiler Chickens, Clostridium perfringens and Enterococcus Counts, and Antibiotic Resistance Phenotypes and Distribution of Antimicrobial Resistance Determinants in Escheric. *Applied and Environmental Microbiology*.73(20): 6566–6576.
- Nihar Ranjan Sarangi, L. K. Babu, A. Kumar, C. R. Pradhan, P. K. Pati and J. P. Mishra (2016). Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens 9(3): 313–319.
- Christian Nordqvis (2017). *Garlic: Proven health benefits. NEWSLETTER.*

- Antti Pönkä, Yvonne Andersson, Anja Siitonen, Birgitta de Jong, Matti Jahkola, Olli Haikala, Aimo Kuhmonen, Pekka Pakkala(1995). Salmonella in alfalfa sprouts. Volume 345, No. 8947, p462–463.
- Jahan Zeb Ansari, Ahsan ul Haq, Muhammad Yousaf, Tanveer Ahmad and Sarzamin Khan (2008). Evaluation of different medicinal plants as growth promoters for broiler. *Sarhad Journal of Agriculture* , 24: 323-330.
- Syeda Q. Ali, Ale Zehra, Baquar S. Naqvi, Shahjahan Shah and Rabia Bushra (2010). Resistance Pattern of Ciprofloxacin Against Different Pathogens. *OMAN MEDICAL JOURNAL*, 25(4): 294–298.

- Jukes ,T. H. (1972). Antibiotics in animal feeds and animal production. *BioSource 22.*, 526-534.
- V. K. Karangiya, H. H. Savsani, Shrikant Soma Patil, D. D. Garg, K. S. Murthy, N. K. Ribadiya, and S. J. Vekariya(2016). Effect of dietary supplementation of garlic, ginger and their combination on feed intake, growth performance and economics in commercial broilers.9(3): 245–250.
- Daniel A. Tadesse, Shaohua Zhao, Emily Tong, Sherry Ayers, Aparna Singh, Mary J.Bartholomew, and Patrick F. McDermot (2005). Antimicrobial resistance in pathogenic Escherichia coli from animal,Antimicrobial resistance in bacteria of animal origin, vol 18(5): 741–749.
- William L. Ragland, Zagreb Biotek d.o.o. Zlatko Janječić, Medicine Ivica Kos (2006), Antibiotic Growth Promoters in Poultry. page-2-5.
- Pan D, Yu Z(2013). Intestinal microbiome of poultry and its interaction with host and diet.;5(1):108-19.

Appendix I

Gadgets

List of gadgets that were used during the study:

Instrument	Manufacturer
Weighing Machine	Adam equipment
UK Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Freezer	Siemens
Vortex Machine	VWR International

Appendix II

Media Composition

The composition of the media used in the present study is given below.

1. Nutrient Agar

Ingredients	**Amount (g/L)**
Peptone	5.0
Yeast Extract	2.5
NaCl	5.0
Agar	15.0

2. MR-VP broth

Ingredients	**Amount (g/L)**
Peptone	7
Dextrose	5
Potassium phosphate	5

3. MacConkey Agar

Ingredients	Amount (g/L)
Peptone	10.0
Manitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

4.TSI Agar

Ingredients	Amount (g/L)
Yeast extract	3.0
Lab-Lemco' powde	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

5.Citrate agar

Ingredients	Amount (g/L)
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000

6.Eosin Methylene Blue Agar

Ingredient	Amount (g/L)
Peptone	10.0
Lactose	5.0
Sucrose	5.0
K_2HO_4	2
Agar	13.5
Eosin	0.5
Methylene Blue	0.06

7.Mueller Hinton Agar

Ingredient	Amount (g/L)
Meat, infusion solids from 300g	2.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000

8.Salmonella Shigella Agar

Ingredient	Amount (g/L)
Beef Extract	5.00
Enzymatic Digest of casein	2.50
Enzymatic Digest of Animal Tissue	2.50
Lactose	10.00
Bile salts	8.50
Sodium citrate	8.50
Sodium thiosuilfate	8.50
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	13.50

Buffers and reagents

1. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4oC.

2. Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4oC.

3. Oxidase reagent

Hundred of N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4oC.

4. Catalase Reagent

From a stock solution of 35 % hydrogen peroxide, 583 µl solutions was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.