# COMPARATIVE EFFICACY OF PROBIOTIC & PREBIOTIC UPON GROWTH PERFORMANCE AND ENTERIC DISEASE IN BROILER

**A THESIS**

**BY**

**MD. MAMUNUR RASHID**
**REGISTRATION NO. 1605175**
**SEMESTER: JANUARY–JUNE, 2017**
**SESION: 2016-2017**

**MASTER OF SCIENCE (MS)**

**IN**

**MEDICINE**

**DEPARTMENT OF MEDICINE, SURGERY & OBSTETRICS**

**HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY DINAJPUR-5200**

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**Submitted to the**
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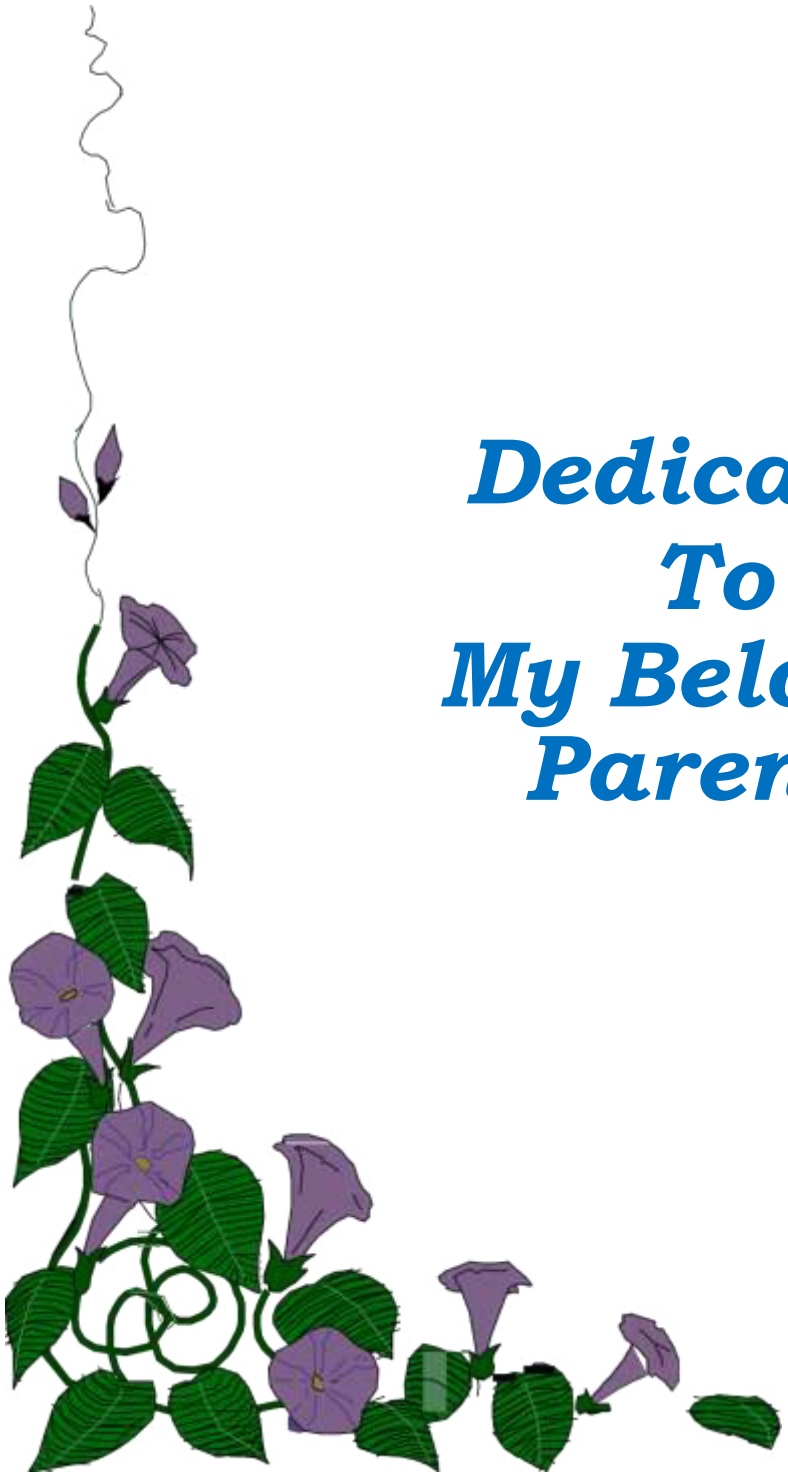

***Dedicated***
***To***
***My Beloved***
***Parents***

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*The Author*

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# ABSTRACT

Commercial poultry production is ranked among the highest source of animal protein in the world. Microbial infections caused by bacteria and parasites particularly *Eimeria* has continued to challenge the poultry industry. Antibiotic Growth Promoters (AGP) have been traditionally used to counter microbial infections in poultry. But due to public health concerns, the use of AGP in poultry is either restricted or out rightly banned in several countries. Hence, this review is aimed at highlighting alternative feed supplements that can enhance performance and protect the chickens from microbial infections. The study found that dietary supplements containing probiotic, prebiotic and enzymes are able to enhance performance while protecting the chickens from microbial infection.

The study was conducted to evaluate the comparative efficacy of probiotic & prebiotic upon growth performance & enteric disease of broiler chickens. The study is conducted at a trial broiler farm in Gazipur districts of Bangladesh for 30 days which was started on the last week of November, 2017 & extended upto the last week of December,2017.Birds (n=450) were randomly and equally distributed into Six groups (Group $A^1$ & $A^2$, $B^1$ & $B^2$, $C^1$ & $C^2$) Group A for Probiotic, B for Prebiotic & C for Control and fed on a diet containing Probiotic & Prebiotic 0.5 kg/MT feed respectively for 4 weeks. The body weight (1772.7±56.89) was significantly increased in the treatment groups fed with probiotics diet from 1$^{st}$ days to 30$^{th}$ days old, then the birds fed with prebiotic (1625±35.80). Weight (1394±62.60) was also significantly decreased among the control groups. . A total of 19 chicks died from Group $C^1$ (Control group) and 11 Chicks at Group $C^2$. Total 20% mortality found in control group. Only two birds died in Group B. In Group A there was no visible morbidity and mortality. The birds which were feed with saltose plus have the lowest FCR (1.34) than the broilers fed with agrimos (1.46) and basal diet (1.67).The study suggests that, the efficacy of probiotic is much stronger than prebiotic but both of these are very helpful for prevention of enteric disease & enhancing the growth of broiler chicken. *Escherichia coli, Salmonella spp, Clostridium perfringens* were the major enteric disease agents found in of the broiler chicken.

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# LIST OF ABBREVIATIONS AND SYMBOLS

| | | |
|---|---|---|
| - | : | Negative |
| # | : | Identifying number |
| % | : | Percentage |
| @ | : | At the rate of |
| + | : | Positive |
| µg | : | Microgram |
| µl | : | Microlitre |
| $^0$C | : | Degree of celcius |
| Ag | : | Antigen |
| Assist | : | Assistant |
| BA | : | BloodAgar |
| BD | : | Bangladesh |
| BGA | : | Brilliant Green Agar |
| EMB | : | Eosin Methylene Blue |
| et al. | : | Associated |
| etc | : | Etcetera |
| FAO | : | Food and Agricultural Organization |
| Gm | : | Gram |
| H.S | : | Haemorrhagic septicemia |
| $H_2O_2$ | : | Hydrogen peroxide |
| $H_2S$ | : | Hydrogen sulphide |
| HSTU | : | Hajee Mohammad Danesh Science and Technology University |
| i.e. | : | That is |
| Ltd | : | Limited |
| MS | : | Master of Science |
| MC | : | MacConkey Agar |
| MI | : | Milliliter |
| MIU | : | Motility Indole Urease |
| MR | : | Methyl Red |
| NA | : | Nutrient Agar |
| NB | : | Nutrient Broth |

# LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

| | | |
|---|---|---|
| No. | : | Number |
| PBS | : | Phosphate Buffer Saline |
| PM | : | Post Mortem |
| Prof. | : | Professor |
| PSS | : | Physiological Saline Solution |
| RPM | : | Rotation Per Minute |
| SC | : | Subcutaneous |
| SE | : | Standard Error |
| SL | : | Serial number |
| Sp | : | Species |
| SSA | : | Salmonella Shigella Agar |
| v/v | : | Volume by volume |
| VP | : | Voges-Proskauer |
| w/v | : | Weight by volume |
| AGP | : | Antibiotic Growth Promoter |
| ADG | : | Average Daily Gain |
| DFI | : | Daily Feed Intake |
| FCR | : | Feed Conversion Ratio |
| AWG | : | Average Weight Gain |

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1

# INTRODUCTION

Over 80% people of the Bangladesh live in the rural areas, and are highly dependent on agricultural system that is finely attuned to a tropical monsoon climate (BBS, 2013). About 49.8% people live below the poverty line and per capita availability of animal protein presently stands at around 21 g meat/day, the recommended intakes of 120 g meat/day. Total meat production in Bangladesh was 11300 metric tons of which chicken and duck meat contributed 154,000 tons. Per capita meat consumption was only 5.12 kg/ year (BBS, 2011) which is markedly below recommended requirements. Meat production could be increased through poultry rearing .The biggest challenge of commercial poultry production is the availability of good quality feed on sustainable basis at stable prices. In spite of this challenge, commercial poultry production ranks among the highest source of animal protein (Iyayi, 2008).With improved stock, broiler birds can attain a weight of 2-3 kg within five to six weeks. However, this production capacity is subject to disease control. With the current advent of excluding antibiotic growth promoters in Bangladesh, the issue of controlling enteric infections without the use of antibiotics becomes challenging. Mortality caused by infection is a major problem in the poultry industry. Such infections are responsible for reduced growth rates. In Poultry, antibiotics are also added to the feed to accelerate the growth of healthy animals. Unfortunately, the long term and extensive use of antibiotics result in selection for the survival of resistant bacteria species or strain (Aarestrup, 1999). Consequently, some countries have banned (Sweden-January 1986) or limited (European union-January 2000, total withdrawal January 2006) the general use of in-feed antibiotics as growth promoters in animals (Montagne *et al*., 2003). Various mechanisms by which AGP acts have been proposed. Firstly, the nutrients are more efficiently absorbed and less are utilized by the gut wall due to a thinner epithelium. Secondly, more nutrients are available to the host because of a reduced intestinal microflora. Thirdly, there is a reduction in harmful gut bacteria which may reduce performance and cause sub clinical infection. Fourthly, production of growth suppressing toxins or metabolites is reduced. But with the a fore-mentioned limitation of AGP the consequent need for their total withdrawal becomes necessary. Hence, this review is aimed at highlighting alternative feed supplements that have probiotic effects and promote growth of broiler chickens, thus achieving both

enhanced performance and good health even without the use of antibiotics. In order to find better alternatives to AGP, research has focused on utilization of feed additives such as enzymes, probiotics, prebiotics, symbiotic products and even nutrition to enhance gut health in poultry and prevent or limit production losses due to enteric infections.

Among bacterial diseases, necrotic enteritis (NE) is one of the most important diseases in poultry that destroys the intestinal lining of the digestive tract, outbreaks occurring in broilers from 2-5 weeks of age. Mortality is usually between 2-10% but can be as high as 40-50%.The incidence of necrotic enteritis in Mymensingh district of Bangladesh is 0.60% (Islam et al., 1998) and 0.52% (Talha et al., 2001). The incidence of necrotic enteritis in Sylhet and Rajshahi Region of Bangladesh is 0.44% (Islam et al., 2003) and 0.91% (Hossain et al., 2002), respectively.

*Escherichia coli* is a common pathogen for commercial poultry causing Colibacillosis all over the world. It is a major cause of respiratory and septicemic diseases in broiler chicken causing mortality less than 5% with morbidity over 50% (Barens and Gross., 1997). The incidence rate of the disease has been reported to be about 22% in Bangladesh (Kamaruddin *et al*., 2007).

At the present time there is a silent killer called "Salmonellosis" which causes constant losses to poultry industry economically and thus makes difficult in the establishing this industry (Khan *et al.,* 1998). Pullorum disease and Fowl typhoid have become wide spread in Bangladesh like other area of the world (Sarker, 1976 and Rahman *et al.,* 1979). Mortality may very from negligible to (10% to 80%) (Kumar and Khaushi, 1988; Kaura *et al,*. 1990; Kleven and Yoder. 1998). The diseases are caused by *Salmonella gallinarum* and *Salmonella pullorum* respectively. Pullorum disease is occurs in first 2-3 weeks of age & Fowl typhoid is frequently in adult birds; there are also reports of high mortality in young chicks (Christensen *et al.,* 1992). *Salmonella gallinarum* is the most devastating disease in Bangladesh (Begum *et al.,* 1993 and Hoque *et al.,* 1992).

The present study was designed to fulfill the following objectives:

- To study the potentiality of Probiotic & Prebiotic against enteric disease in broiler chickens.
- To compare the efficacy of Probiotic & Prebiotic in broiler in terms of body weight gain, daily feed intake and Feed Conversion Ratio (FCR).
- To find better alternatives to Antibiotic Growth Promoter (AGP)

# CHAPTER 2

## REVIEW OF LITERATURE

# CHAPTER 2

# REVIEW OF LITERATURE

The purpose of this chapter is to provide a selective review of the past researches conducted in relation to the present research work. The reviews are briefly presented under the following heading and subheading.

## 2.1 Review on Effect of probiotic upon broiler

### 2.1.1 Review of Effect of probiotic on Growth Rate and Body Weight Gain

**Chapman and Lyons, 1989; Buche *et al*., 1992; Holoubek, 1993; Sharma and Katoch, 1996; Ghabdan, 1998),** It can be inferred from various studies that supplementation of probiotics to broiler diet improve body weight gain in broilers. Probiotics also improve general health of chicks.

**Krecov and Puijic (1975),** fed LBA (*streptococcus faecium*) at 200g/400kg feed by they observed that total body weight gain in birds fed with and without LBA were 1570 and 1545 g, respectively.

**Kim *et al.* (1990)** observed increased weight gain of chicken offered maize diet supplemented with probiotics.

**Mohan (1991)** reported improve in growth rate in broilers after feeding probiotics.

**Moses (1992)** reported that when probiotics and antibiotics were used in combination, the body weight at seventh week was 1.6 kg against 1.2 kg in control.

**Khan *et al.* (1992)** observed no increase in body weight gain in broilers after supplementing probiotics to broiler diet.

**Chiang and Hsiegh (1995)** observed better weight gain ($P \leq 0.01$) in birds fed with probiotics-supplemented diet (*Bacillus* and *Streptococcus*) as compared to the probiotics-unsupplemented diets.

**Samanta and Biswas (1995)** reported that supplementation probiotics in drinking water of broiler housed in battery cage observed increase in body weight at starter and finisher phase.

**Jin *et al.* (1996)** reported significant (P≤0.05) higher weight gain in broilers given diets supplemented with *Bacillus subtilis* and *Lactobacillus* culture.

**Mohan *et al.* (1996**) reported increased body weight in broilers fed probiotics at 75 mg/kg feed.

**Jin *et al.* (1997)** reported improved growth performance in broiler after addition of probiotics in feed.

**Bandy and Risam (2001)** conducted an experiment to determine the efficiency of probiotic (Biospur) at three different levels- 25, 50 and75 g/100kg feed respectively and observed that chicks fed with probiotics grew faster than control and highest live weight was obtained in the treatment fed probiotics at 75/100kg feed.

**Anjum *et al.* (2005)** reported that multi-strain probiotics supplementation in the diet significantly (P≤0.05) improved body weight gain in broilers.

**Das *et al.* (2005)** reported no significant (P≤ 0.05) difference in dressed weight and blood parameters in broilers after supplementation of commercial probiotics preparation.

**2.1.2 Effect of probiotic on Feed Intake and Feed Conversion Efficiency**

**Hertrampf (1979)** reported that probiotics had been successful in improving feed conversion efficiency in broilers.

**Cho *et al.* (1992)** reported that the supplementation of *Lactobacillus casei* improved feed conversion ratio by 0.3 to 3.1% as compared to control and treatments supplemented with antibiotics or other probiotics.

**Moses (1992)** reported that supplementation of probiotics product Biospur in the diet of broiler resulted in improved feed conversion efficiency (2.18 vs 2.9) at $17^{th}$ week of age.

**Khan *et al.* (1992)** observed no increase in feed intake and feed conversion efficiency in broilers after supplementing probiotics to the broiler diet.

**Cavazzoni *et al.* (1993)** reported 6 % improvement in feed conversion efficiency in broilers fed diet supplemented with probiotics (*Bacillus sp*) as compared to control.

**Samanta and Biswas (1995)** reported that supplementation of probiotics in drinking water of broilers housed in battery cage increased feed intake during both starter and finisher phases.

**Chiang and Hsiegh (1995)** observed that in hen broiler chickens fed diets supplemented with six probiotics levels up to 6 weeks of age, weight gain: feed ratio was improved with reduction in ammonia production in litter.

**Takahashi *et al.* (1997)** observed improvement in feed conversion efficiency in broilers fed with *Bacillus cereus* even when chicken were raised in unhygienic conditions but, no improvement in live weight gain or feed intake was noticed.

**Rajmane and Sonawane (1998)** reported that when broilers were treated with probiotics through water at 20g/1000 chicks for first five days and from sixth day onwards at 50g/ton of feed till the end of $42^{nd}$ day of age, the better performance in term of FCR as compared to control was noticed.

**Singh and Sharma (1999**) studied the effect of different levels (0.02, 0.03 and 0.04%) of probiotics on commercial broilers and observed highest feed efficiency in broilers offered 0.02 % probiotics.

**Mahajan *et al.* (1999)** studied the effect of probiotics feeding during summer and winter on growth performance and carcass quality of broilers. Feed intake and feed conversion ratio on cumulative basis were significantly ($P \leq 0.05$) higher in probiotics fed broilers Naik *et al.* (2000) valuated the effect of different probiotics.

**Safalaoh *et al.* (2001**) showed that effective microorganisms (probiotics) improved feed efficiency in broilers alone or with antibiotics, which is more pronounced at the higher dosage (30g/kg feed).

**Upendra and Yathiraj (2002)** observed that supplementation of probiotics at 250g/ton of feed resulted in an improvement of FCR, which was 10.8% better over that of control.

**Gupta (2003)** supplemented broiler diets with different strains of *Lactococci* and Bacitracin. He observed that all the diets showed lower ($P \leq 0.05$) FCR than control.

**Chitra *et al.* (2004**) reported that inclusion of probiotics and ascorbic acid both independently and simultaneously either in feed or in drinking water to broilers had made significant ($P \leq 0.01$) improvement in total feed consumption and feed efficiency during summer season.

**Gupta (2004)** also observed that supplementation of probiotics improved FCR in broilers at the field level.

**Anjum *et al.* (2005)** observed that there was significant (P≤ 0.05) improvement in feed conversion ratio after supplementation of multi-strain probiotics in broilers, however, no improvement in feed intake was observed.

### 2.1.3 Effect of probiotic on Survivability in GIT

**Hussein and El-Asry (1991)** found that administration of a *Lactobacillus* concentrates at 0.5g/kg starter mixture to broiler chickens decreased the incidence of diarrhea and mortality.

**Talukdar (1992)** reported non-significant (P≤ 0.05) difference between probiotics treatments; however, marginally lower mortality was observed in the treatments fed pure *Lactobacilli* culture.

**Adsul (1993)** recorded total cumulative mortality of 3.05, 4.14, 3.04 and 4.19% in the birds fed pure *Lactobacilli* culture, enzyme feed supplement, *Lactobacilli* culture with enzyme supplement and control, respectively.

**Lee *et al.* (1994)** noticed that the viability of the broiler treatments given antibiotics and probiotics was higher than that of the control treatment, which was statistically non-significant (P≤ 0.05).

**Bhatt *et al.* (1995)** observed favorable effect on the livability of broilers after addition of probiotics in the diet of broilers.

**Kaistha *et al.* (1996)** recorded lesser mortality in broilers fed diets supplemented with *Lactobacillus acidophilus*, *Streptococcus uberis and Saccharomycs cerevisiae*.

**Samantha and Biswas (1997**) observed that mortality was reduced in broilers fed diet supplemented with probiotics.

**Bhatt *et al.* (1997)** reported the effect of dietary supplementation of different strains of *Streptococcus lactis* on the biological performance of commercial broilers ('starbo' broilers) and found that $S_1$ strain from CFTRI, mysore, showed minimum chick mortality during both starter and finisher phases.

**Rajmane and Sonawane (1998)** reported reduction in chick mortality from 7 to 2 per cent oral administered probiotics through drinking water at 20g/ 1000 chicks.

**Singh *et al.* (1999**) investigated the influence of *probiotic* on mortality of broiler chicks in summer and found that addition of probiotics decreased the mortality.

**Mahajan *et al.* (1999**) reported lower cumulative mortality in probiotic fed broiler chicks in summer and winter when compared with control treatment.

**2.2 Review on Effect of prebiotic upon broiler**

**Cox et al., 2010). Zhang et al. (2008**) found that β-glucans derived from the yeast *S. cerevisiae* significantly increased BW and BWG of broilers at 50 and 75 mg/kg in the diet.

**Rathgeber et al. (2008)** observed enhanced BW in broiler chicks during the grower phase of production.

**Huff et al. (2006)** reported a decrease in performance after β-glucan supplementation in chickens.

**Huff et al. (2006**) found, however, that broiler chicks fed β-glucan for 7 d before an *E. coli* infection had higher BW than challenged birds fed a control diet. Correlating with our findings, several studies have noted no significant effects of β-glucans on growth performance, suggesting that β-glucans do not negatively affect performance in nonchallenge

**Patterson and Burkholder (2003)** reported that fructo-oligosaccharide products such as inulin, fructooligosaccharide and oligofructose are the dominant prebiotics in use today. However, other macromolecules are increasingly being investigated for their prebiotics activities including mannan oligosaccharides.

Birds fed with the diet containing MOS and the diet containing antibiotic showed higher feed intake during the different production stages. They also had higher daily weight gain during the stages 1-10 days of age and 1-28 days of age, which may be attributed to the higher feed intake. During the stage of 1-42 days of age, birds fed SCCW improved daily weight gain, reaching the same daily weight gain and feed conversion ratio as the birds fed MOS and antibiotics.

**Gibson & Roberfroid (1995),** evaluating the use of indigestible carbohydrates, such as plant and yeast cell wall classified as MOS complexes (glucomannanproteins, and particularly mannanoligosaccharides) , found that carbohydrates can bind to the fimbria of bacteria, thus inhibiting the colonization of the gastrointestinal tract by pathogenic microorganisms.

**Chen *et al*., 2005). Yang *et al.* (2008)** studied the effect of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic *E. coli* challenge

**Bauer *et al*., 2006;** reported about Several carbohydrates that may be fermented by intestinal microorganisms can be classified as prebiotics including NSPs, resistant starch and non-digestible oligosaccharides. Inulin and fructo-oligosaccharides are widely used as prebiotic feed additives

**Steiner, 2006;** reported Prebiotics may enhance the digestibility and performance parameters by creating the favourable conditions for beneficial bacteria (

**Fritts, Waldroup, 2003**; reported prebiotics used in a mixture for fattening chickens does not affect the quality of meat chemical composition expressed (

**Rechman, et.al., 2007** reported about a very important group of prebiotics are beta-glucans, which are selected polysaccharides also derived from cell walls of certain strains of yeasts, bacterias and fungi, as well as from algae and cereals (oats, barley). Obtained from mannoproteins by hydrolysis, are not degradated by glucanase, so they can be used in the presence of feed enzymes. In combination with beta-glucans, mannanooligosaccharides positively influence on defense mechanisms of the digestive system and neutralization of pathogens, they activate digestive enzymes and improves nutrients absorption from the diet. Broiler chickens fructooligosaccharides supplemented diet significantly increases the number of bifidobacteria and lactic acid bacteria in the cecum and small intestine. Furthermore, population of *Clostridium perfringens*, *Escherichia coli* and *Salmonella* are significantly reduced. FOS alleviates the effects of caecal epithelial necrosis, and also stimulates growth of intestinal villi and crypts in the jejunum and iliac colon.

## 2.3 Isolation, identification and characterization of Enteric Microorganism

### 2.3.1 Isolation, identification and characterization of *Escherichia coli* from chicken

**AI-Ghamdi *et al*. (2001)** studied the identification of common bacterial organisms in locally produced healthy chickens. Among the isolated 28 types of bacterial organisms, *E. coli* was the most common (16.2%) cases.

**Chau *et al*. (2002)** isolated *E. coli* from chickens and eggs. Of 47 *E. coli* isolates, 36 were from chickens and 11 were from eggs. The isolates were identified as *E. coli* belonging to the 01:K1, 02:K l and 078:K80 serotypes.

**Derakhshantar and Ghanbarpour (2002)** studied avian cellulites in broiler chickens. The authors identified 91.8% of *E. coli* infection from cellulites of broiler by bacteriological investigation

**Dho-Moulin *et al.* (1999)** stated that avian pathogenic *E. coli* (APEC) cause airsacculitis, polyserositis, septicemia and other mainly extra intestinal diseases in chickens, turkeys and other avian species. APEC are found in the intestinal microflora of healthy birds and most of the diseases associated with them are secondary to environmental and host predisposing factors. APEC isolates commonly belonged to certain serogroups, O1, O2 and O78.

**Ewers *et al*. (2004)** investigated molecular biology and epidemiology of 150 avian pathogenic *Escherichia coli* strains (APEC) isolated from septicemic poultry in Germany by serotyping, pulsed field gel electrophoresis (PFGE), and polymerase chain reaction (PCR). Only 49.6% of the isolates were grouped to serogroups O1, O2, and O78.

**Guan *et al.* (2002)** identified *E. coli* by the analysis of 16S rRNA gene by PCR.

**Hossain *et al*. (2008)** isolated *E. coli* from apparently healthy broilers and layers from different poultry farms adjacent to the Bangladesh Agricultural University. A total of 110 fecal samples were collected from broiler (n=55) and layer (n=55) chickens. *E. coli* were isolated and identified by cultural, biochemical, motility test and the heat-stable toxins were determined by Infant Mouse Assay (IMA). In case of broilers, 35 (63.6%) samples were found positive while 31 (56.4%) from layers. The overall prevalence of *E. coli* was 60%.

**Kalin *et al.* (2012)** investigated the presence of *E. coli* O157 and its virulence genes in various samples collected from broiler chickens and humans in Eastern Turkey by culture, immunomagnetic separation (IMS), and polymerase chain reaction . In the PCR *E. coli* O157 was identified in 0.1% (1/1000) and 0.4% (4/1000) of the liver and cecum samples of broiler chickens, respectively. On the other hand, none of the carcass samples were determined to be positive for *E. coli* O157. Overall, the results indicated that 12% (3/25) of the flocks were

**Pandian (2006)** isolated 25 avian pathogenie *E. coli* (APEC) from 105 heart blood samples from birds in Tamil Nadu, India. They identified that the isolates were as belonging to the 01, 02, 015, 018, 024, 053, 060, 091, 0103, 016 and 0169 serotypes, respectively. Positive for *E. coli* O 157.

**Roy *et al.* (2006)** isolated *E. coli* from Japanese quail and their environment. Of 31 *E. coli* isolates, 11 were from heart blood of dead Japanese quail and 20 were from dead in shell embryos, fluff sample, foot bath and drinking water.

**Ali *et al.* (1998)** described the colony characters of *E coli*. They found metallic sheen on the EMB agar, rose pink colony on the MacConkey agar and pinkish colony on the SS agar media.

**Hossain *et al.* (2008)** observed that *E. coli* shows characteristic yellow green metalic sheen on EMB agar, whereas on MacConkey agar and SS agar, bright pink or red colonies and pinkish colonies were seen respectively.

**Sharada *et al.* (1999)** reported that the *E. coli* colonies on nutrient agar are smooth, moist and low convex. The colonies on EMB agar appear dark with characteristics metallic sheen.

**Dey *et al.* (2013)** stated that pink colored, rod shaped, short chain, single or paired Gram negative bacilli were observed in *E. coli* isolates from pigeon after Gram's staining.

**Hossain *et al.* (2008)** observed the staining characters of *E. coli* as a gramnegative, short rod, single, pair or in short chain.

**Merchant and Packer (1967)** described the staining characters of *E. coli* as a gram-negative, short rod, varying from coccoid bipolar shape to long filamentous form.

**Mishra *et al.* (2002)** stained *E. coli* from domestic poultry and found short rod to coccoid shape bacteria.

**Adeyanju and Ishola (2014) c**onducted that catalase test, sugar fermentation using TSI (LabM, Uk), Kovac's test for identification of *E. coli* isolates.

**Ahmad *et al.* (2009)** identified 26 isolates of *E. coli* on the basis of indole test and biochemical reaction.

**Ali *et al.* (1998)** reported the fermentation tests of *E. coli*. The fecal isolate fermented four sugars except maltose whereas urinary isolate fermented all the four sugars (Dextrose, lactose, sucrose and mannitol).

**Collins and Lyne (1976)** described the indole formation by *E. coli* with kovac's reagent which was a rose pink color.

**Edward *et al.* (1968)** conducted some tests with *E. coli* and concluded that *E. coli* produced indole, gave a negative Voges-Proskauer reaction and did not utilize citrate as the sole carbon source. Isolates of these genera usually did not produce extracellular DNase, H2S, phenylalanine deaminase, urease, and did not grow in inositol and KCN containing media. Typical gas production from glucose, indole, lysine, arabinose, mannitol, ortho-nitrophenyl galactocide (ONPG), trehalose and xylose was found in this genus.

**Joshi *et al.* (2012)** performed indole, methyl red, Voges-Proskauer, Simon's citrate test (IMViC), catalase, oxidase, urease, H2S production in TSI and sugar fermentation test for biochemical characterization of *E. coli.*

**Khaton *et al.* (2008)** stated that isolates of *E. coli* fermented dextrose, lactose, maltose and mannitol with the production of acid and gas but did not ferment inositol. Acid production was indicated by the change from reddish to yellow and gas production by the accumulation of gas bubbles in the inverted Durham's tube.

**Thomas (1988)** performed some biochemical tests for *E. coli* and observed that most *E. coli* fermented lactose, reduced nitrates and were methyl red positive. Approximately 10% of the species were late lactose fermenters; and some of them were non lactose fermenters.

### 2.3.2 Isolation, identification and characterization of *Salmonella. spp.* from chicken

**Ellerbroek *et al.,* (2010)** isolated *Salmonella* from 400 imported chicken carcasses in Bhutan and from 178 pig carcasses in Vietnam for antibiotic resistance analyzed on a random basis against 14 antimicrobial agents. Among the poultry samples tested, 13% were positive for *Salmonella*.

**Ahmed *et al.,* (2009)** isolated 69 *Escherichia coli* and 10 *Salmonella*, from retail chicken meat in Hiroshima prefecture, Japan, the samples were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes.

**Akter *et al.,* (2007)** determined the seroprevalence of Salmonellosis in layer flocks and antibiogram study following isolation of *Salmonellae*. This study was conducted during the period from January to May 2006 at Gobindapur of Dinajpur district. A total of 225 Star cross 579 brown chickens were studied with rapid serum plate agglutination test. Liver of 200 dead birds was studied for isolation and identification of *Salmonellae*. In vitro antibiotic sensitivity test of isolated *Salmonellae* was performed with commercial sensitivity discs. The overall seroprevalence was recorded 23.11%. The prevalence was varied from age to age. The highest rate was 28% in above 20 weeks of age. The antibiogram study revealed that the isolates were sensitive to ciprofloxacin (80%), nitrofurantoin (100%), sulphamethoxazole/trimeoprim and amoxycillin (50%), tetracycline (60%) but resistant to penicillin-G and erythromycin. The author suggest for further studies on serotyping of the isolated *Salmonellae*, isolation and identification of *Salmonellae* from different feed and environmental samples.

**Roy *et al.,* (2002)** isolated five hundred sixty-nine *Salmonella* out of 4745 samples from poultry products, poultry, and poultry environment in 1999 and 2000 from the Pacific Northwest. These *Salmonella* were identified to their exact source, and some were serogrouped, serotyped, phage typed, and tested for antibiotic sensitivity.

**Muktaruzzaman *et al.*, (2010)** mentioned that *Salmonella* organisms showed different cultural characteristics in different media. These were turbidity in Tetra Thionate broth, pink white color colonies in Brilliant Green agar, gray white colony in Nutrient agar, slightly grayish color colonies in *Salmonella*-Shigella agar, black color colony in Tripple Suger Iron agar, pale color colonies in MaConkey's agar, well defined glistening colonies in Blood agar and pinkish colonies in EMB agar.

**Hossain (2002)** isolated *Streptococcus aureus, Escherichia coli* and *Salmonella* from diarrhoeic calves. The author stated that the *Salmonella spp.* produced small round and smooth colonies on nutrient agar and opaque, translucent and colorless colonies on SS agar. The organisms produced colourless, pale, transparent colonies on MacConkey agar and small, round, low convex, translucent, pale red colour colony on BGA against pinkish background which was initially green in colour.

**Freeman (1985)** stated that the optimum growth temperature of *Salmonella* is 37 _C, but good growth is observed at room temperature.

**Freeman (1985)** stated that *Salmonella typhi* produce "maple leaf" like irregular margin and slightly roughened glistening surface colony on nutrient agar.

**Cheesbrough (1985)** noted that on SS and MC agar *Salmonella* produce lactose nonfermenting colony. Most strains shows blackening of the colony due to H2S Production.

**Gene O (2002)** reported the rod and short to long chain forming *Salmonella* organisms, which were isolated and identified from liver, spleen, intestinal contents of animals and birds.

**Cheesbrough and Freeman (1985)** reported that *Salmonellae* are gram negative bacilli whose cellular morphology closely resembles and indistinguishable from other enterobacteria.

**Freeman (1985)** showed Giemsa stain from a pure culture of *Salmonella* reveals that the organisms are rod shaped, single of paired in arrangement.

**Sujatha *et al.,* (2003)** isolated *S. gallinarum* from poultry in and around Hyderabad and Secunderabad cities in India and characterized. All isolates showed positive reaction to MR, citrate and H2S. Sugar fermentation tests revealed acid production occur without gas from glucose, maltose, dulcitol, galactose, trehalose, xylose and rhamnose.

**Lee *et al.,* (2003)** reported that Fowl typhoid (FT) in Korea is caused by *Salmonella gallinarum*. The authors investigated the biochemical characteristics and antimicrobials susceptibility of field isolates of *S. gallinarum* isolates in Korea. A total of 258 isolates of *S. gallinarum* from 1995 to 2001 showed the same pattern in the majority of biochemical test such as IMViC (indole, methyl red, Voges-Proskauer and citrate

utilization), carbohydrate fermentation and amino acid decarboxylation, and these results were almost in accordance with the traditional biochemical characteristics of *S. gallinarum* strain. Therefore, results indicate that sorbitol fermentation and arginine decarboxylation showed the diversity by isolates and the vast majority of isolates from 2001 showed the reduced susceptibility to antimicrobials tested.

**Hossain (2002)** conducted an experiment that the *Salmonella* when allowed to ferment the five sugars were found to ferment dextrose, maltose and mannitol with the production of acid and gas but no fermentation was observed in lactose and sucrose.

**Buxton and Fraser (1977)** stated that the *Salmonellae* did not ferment lactose, sucrose or salicin but did ferment glucose, maltose, mannitol, dulcitol and dextrin with production of acid and gas. These were also characterized by their ability to reduce nitrates to nitrites and to produces H2S. The authors could not decompose urea, liquefy gelatin or produce indole.

**Dhruba *et al.*,** (2000) isolated the *Salmonella* organism using five basic sugar fermentation reactions. Nearly all isolates were positive for mannitol, maltose and dextrose with the production of acid while lactose and sucrose are not fermented.

### 2.3.3 Isolation, identification and characterization of *Clostridium sp.* from chicken

**Eyre, 2009 ;** reported that Sugar such as mannitol, sucrose, maltose and lactose were prepared at 1% concentration. Five (5) milli litres was dispensed into test tubes and autoclaved. They were inoculated with the test organism and incubated aerobically at 370C for 48 hours, followed by observation for change in colour.

**Jeffrey and Stanley, 2001** studied and reported that Egg yolk emulsion (2ml) was mixed with nutrient broth and 1% Sodium chloride added for clearance of the media. The inoculating loop was thrusted with the sample into the egg yolk media and was incubated anaerobically for 24 hours at 370C

**Rahman 1995 described that** Impression smears from 50 samples prepared from jejunum of intestine were stained with Gram's staining method**.**

# CHAPTER 3

## MATERIALS AND METHODS

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Study area and period

The research work was carried out on a trial broiler farms in Gazipur .The laboratory works were conducted in the Microbiological Laboratory of AG Agro Industries, Gazipur Sadar, Gazipur, Bangladesh, during the period from 20th November, 2017 to 19th December, 2017.

### 3.1.2 Experimental Group

The chicks (n = 450) at day old were divided into 6 equal groups (Group A ¹ = 7 5 & A ² = 7 5 , B ¹ = 7 5 & B ² = 7 5 , C ¹ = 7 5 & C ² = 7 5 ). Group A for Probiotic, B for Prebiotic & C for Control. Each group contain 75 birds.

### 3.1.3 Cleaning and Disinfection of farm house

Poultry Shed were cleaned by Using a broom, brush & shove to remove dust, soil and dry organic material. All items of required Equipment including Hover, Chick Guard, Feeder, Waterer, cleaned with washing detergent solution Surf Excel®) .Contaminated Equipment, floor, ceiling fan,wall was disinfected with N-alkyl dimethyl benzyl ammonium chloride 40% & Urea 60% solution prior to cleaning. The Feeder & Waterer were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized by direct sunlight.

### 3.1.4 Management of birds

#### 3.1.4.1 Brooding

Brooding Temperature:

| Week | Temp (°F) |
|---|---|
| 1st | 95 |
| 2nd | 90 |
| 3rd | 85 |
| 4th | 80 |

### 3.1.4.2 Vaccination

| Age (Day) | Name of Vaccine | Route of Administration |
|---|---|---|
| 3rd | BCRDV (CEVAC NEW-L) | Eye Drop |
| 11th | IBD (CEVAC IBD-L) | Drinking Water |
| 18th | IBD (CEVAC IBD-L) | Drinking Water |
| 22th | RDV (CEVAC NEW-L) | Drinking Water |

Fig-1: Research Farm

Fig-3 Vaccination of birds at day 3


Fig-2: Washing Soda (Surf Excel)


Fig-4: Disinfectant (Timsen)

### 3.1.4.3 Feeding

1. Birds were weighed (100%) at day old, at the end of starter (14 days of age), grower periods for each brand of feeds, and feed consumed for each type of feed were recorded accurately.

Fig-5: Experimental Group

Fig-6: Individual flock

Fig-7: Body weight at the end of starter


Fig-8: Body weight at the end of Grower

2. The feeds used for this trial were pretreated with Probiotics (Saltose Plus-Opsonin Pharma Limited & Preboitics (Agrimos- Lallemand Animal Nutrition).

3. Feed was given in the morning and evening and that were refused from the previous day, removed before the new meal was given on the following morning. However, for this research Three rations were used (1) basal diet, (2) basal diet+ Saltose plus (3) Basal diet+ Agrimos.

4. Starter is fed at day old to day 14 & Grower is fed at day 15 to day 30 of birds age.

## 5. Composition of Probiotic (Saltose Plus) :

**Trade Name**: Saltose Plus®

**Source:** Opsonin Pharma Bangladesh Limited.

**Composition:**

- Bacillus licheniformis---- More than 1.0*106 CFU.
- Bacillus subtilis. ---------- More than 1.0*106 CFU.
- Bacillus pumilus. ---------- More than 1.0*106 CFU.
- Enterococcus faecalis. ------ More than 1.0*106 CFU.
- Enterococcus faecium. --- More than 1.0*106 CFU.
- Beta- Xylanase----------350 U
- Cell-wall Lyase----------3,700 U
- Protease----------12,000 pU

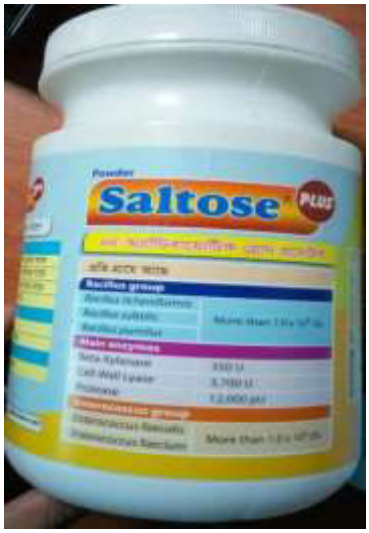


Fig-9: Probiotic + Enzyme

## 6. Composition of Prebiotic (Agrimos):

**Trade Name**: Agrimos®

**Source:** Rx Nutrivet Bangladesh Limited (Lallemand Animal Nutrition).

**Composition:**

- Mannans Oligo-Saccharide (MOS)--- 26%.
- Beta-Glucans. ---------- 27%.

Fig-10: Prebiotic

## 7. Feed Composition

**Table 1: Composition of Broiler Starter (100 Kg)**

| Sl. No. | Particulars | Quantity/kg (Probiotic) | Quantity/kg (Prebiotic) | Control |
|---|---|---|---|---|
| 1 | Maize | 50.090 | 50.090 | 50.140 |
| 2 | Soyabean Meal | 30.803 | 30.803 | 30.803 |
| 3 | Soya Fullfat | 5.900 | 5.900 | 5.900 |
| 4 | Rice bran oil | 2.350 | 2.350 | 2.350 |
| 5 | Protein Meal | 6.000 | 6.000 | 6.000 |
| 6 | Rice Polish | 2.500 | 2.500 | 2.500 |
| 7 | Lime Stone Powder | 0.900 | 0.900 | 0.900 |
| 8 | DCP (Di-Calcium-Phosphate) | 0.200 | 0.200 | 0.200 |
| 9 | DL Methionine 99% | 0.250 | 0.250 | 0.250 |
| 10 | Salt | 0.240 | 0.240 | 0.240 |
| 11 | Vitamin Premix | 0.050 | 0.050 | 0.050 |
| 12 | L-Lysine 79% (Hcl) | 0.100 | 0.100 | 0.100 |
| 13 | Sodium Bi Carbonate | 0.070 | 0.070 | 0.070 |
| 15 | Toxin binder | 0.120 | 0.120 | 0.120 |
| 16 | Mould Inhibitor | 0.100 | 0.100 | 0.100 |
| 17 | Mineral Premix | 0.050 | 0.050 | 0.050 |
| 18 | L-Threonine 98% | 0.080 | 0.080 | 0.080 |
| 19 | Emulsifier | 0.050 | 0.050 | 0.050 |
| 20 | Coccidiostate (Decoquinate 6 %) | 0.060 | 0.060 | 0.060 |
| 21 | Phytase | 0.010 | 0.010 | 0.010 |
| 22 | Xylanase | 0.020 | 0.020 | 0.020 |
| 23 | Antioxidant | 0.007 | 0.007 | 0.007 |
| **24** | **Agrimos/Saltose Plus** | **0.050** | **0.050** | |
| | Total | 100.000 | 100.000 | 100.000 |

**Nutrient Limit**

| | |
|---|---|
| C. Protein | 23.00% |
| C. Fat | 6.53% |
| C. Fiber | 3.86% |
| Calcium | 1.20% |
| Phosphorous | 0.58 % |
| ME | 3,072.77 Kcal/kg |

**Table 2: Composition of Broiler Grower (100 Kg)**

| Sl. No. | Particulars | Quantity/kg (Probiotic) | Quantity/kg (Prebiotic) | Control |
|---|---|---|---|---|
| 1 | Maize | 51.190 | 51.190 | 51.240 |
| 2 | Soyabean Meal | 27.800 | 27.800 | 27.800 |
| 3 | Soya Fullfat | 6.800 | 6.800 | 6.800 |
| 4 | Rice Bran oil | 3.400 | 3.400 | 3.400 |
| 5 | Protein Meal | 6.000 | 6.000 | 6.000 |
| 6 | Rice Polish | 2.463 | 2.463 | 2.463 |
| 7 | Lime Stone Powder | 0.900 | 0.900 | 0.900 |
| 8 | DCP (Di-Calcium-Phosphate) | 0.200 | 0.200 | 0.200 |
| 9 | DL Methionine 99% | 0.240 | 0.240 | 0.240 |
| 10 | Salt | 0.240 | 0.240 | 0.240 |
| 11 | Vitamin Premix | 0.050 | 0.050 | 0.050 |
| 12 | L-Lysine 79% (Hcl) | 0.100 | 0.100 | 0.100 |
| 13 | Sodium Bi Carbonate | 0.070 | 0.070 | 0.070 |
| 14 | Toxin Binder | 0.120 | 0.120 | 0.120 |
| 15 | Mould Inhibitor | 0.100 | 0.100 | 0.100 |
| 16 | Mineral Premix | 0.050 | 0.050 | 0.050 |
| 17 | L-Threonine 98% | 0.080 | 0.080 | 0.080 |
| 18 | Emulsifier | 0.050 | 0.050 | 0.050 |
| 19 | Coccidiostate (Decoquinate 6 %) | 0.060 | 0.060 | 0.060 |
| 20 | Phytase Enzyme | 0.010 | 0.010 | 0.010 |
| 21 | Xylanase Enzme | 0.020 | 0.020 | 0.020 |
| 22 | Antioxidant | 0.007 | 0.007 | 0.007 |
| **23** | **Agrimos / Saltose Plus** | **0.050** | **0.050** | |
| | Total | 100.000 | 100.000 | 100.000 |

**Nutrient Limit**

| | |
|---|---|
| C. Protein | 22.43% |
| C. Fat | 7.71% |
| C. Fiber | 3.75% |
| Calcium | 1.19 % |
| Phosphorous | 0.58 % |
| ME | 3,161.71 Kcal/Kg |

### 3.1.5 Microbiological study for isolation and identification of enteric organism

The media and reagents that have been used for the isolation and identification of the bacteria are mentioned below.

#### 3.1.5.1 Solid media

- Nutrient Agar Medium, (HI-MEDIA, India)
- Blood Agar Medium, (HI-MEDIA, India)
- Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
- Brilliant Green Agar Medium, (HI-MEDIA, India)
- Mac Conkey Agar medium, (HI-MEDIA, India)

#### 3.1.5.2 Liquid media

- Nutrient broth, (HI-MEDIA, India)
- Methyl Red-Voges Proskauer (MR-VP) broth, ( HI-MEDIA, India)

#### 3.1.5.3 Media for bichemical test

- Simmons Citrate Agar Medium, (HI-MEDIA, India)
- Methyl Red (MR) media
- Pepton broth, (HI-MEDIA, India)
- Lactose broth, (HI-MEDIA, India)
- Voges-Proskauer (VP) media

### 3.1.6 Reagents

The chemicals and reagents used during the study were-

- Gram's staining reagents (Crystal violet, Gram's iodine, Acetone alcohol, Safranin)
- Potassium- di-hydrogen phosphate (0.2M, $KH_2PO_4$ $2H_2O$)
- Methylene Blue stain
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)

- Dehydrated sodium citrate
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents as when required during the experiment.
- Indol Solution
- Methyl Red Solution
- Voges-Proskauer (VP) Solution

### 3.1.7 Glassware's and appliances

The different kinds of glassware's and appliances that were used as follows:

Test tubes (with or without Durham's fermentation tube and stopper).

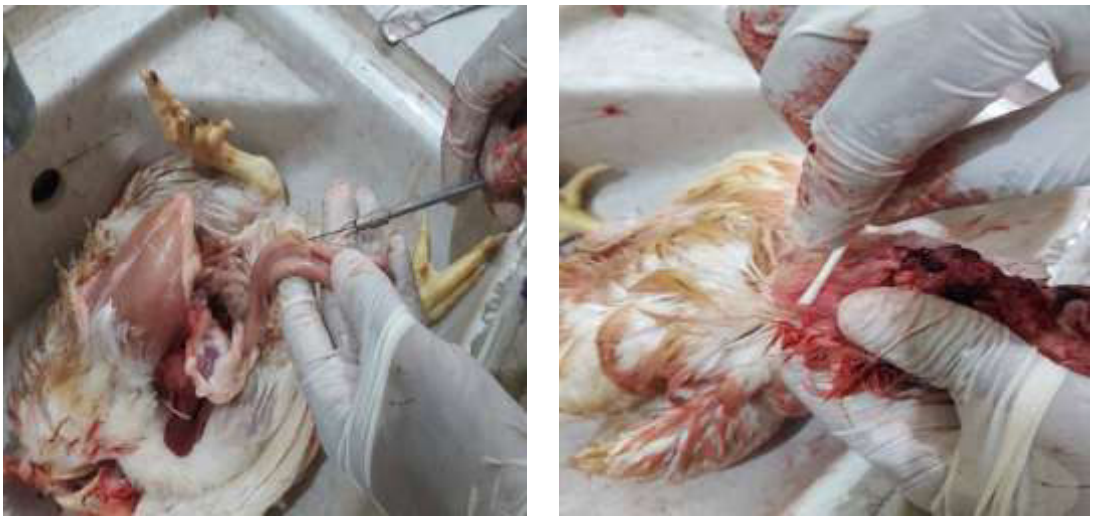
- Conical flask
- Inoculating loop
- Petridishes
- Pipette
- Cover slips
- Hanging drop slide
- Glass rod spreader, Slide
- Test tube stand Water bath
- Ice box
- Autoclave
- Refrigerator
- Hot air oven
- Compound microscope
- Micropipette
- Centrifuged tube
- Spirit lamp

### 3.2 Methods

The experimental work was divided into two steps: The first step was performed for the observation & recording of Growth Performance & Mortality of Broiler chicken . The second step was conducted for the isolation and identification of the organisms of *Clostridium Perfringens*, *Escherichia coli* & *Salmonella Spp*. by clinical Diagnosis & through the collected sample using cultural, staining and biochemical characteristics for detecting the immune response against those organism into the probiotic & prebiotic treated flock.

#### 3.2.1 Samples for the isolation of bacteria

Samples like liver, Tracheal fluid, and intestinal contents were collected from affected chickens.



**Intestinal Fluid** **Tracheal Fluid**

**Fig 11: Sample Collection**

#### 3.2.2 Culture and morphological staining of bacteria

Using standard bacteriological procedures, the isolation and identification of the isolated colonies in different culture media was performed as described by Buxton and Fraser (1977). By using Gram's stain described by Merchant and Packer (1967) ,the representative bacterial colonies were characterized morphologically.

The layout of the diagrammatic illustration of the present study is shown in figure

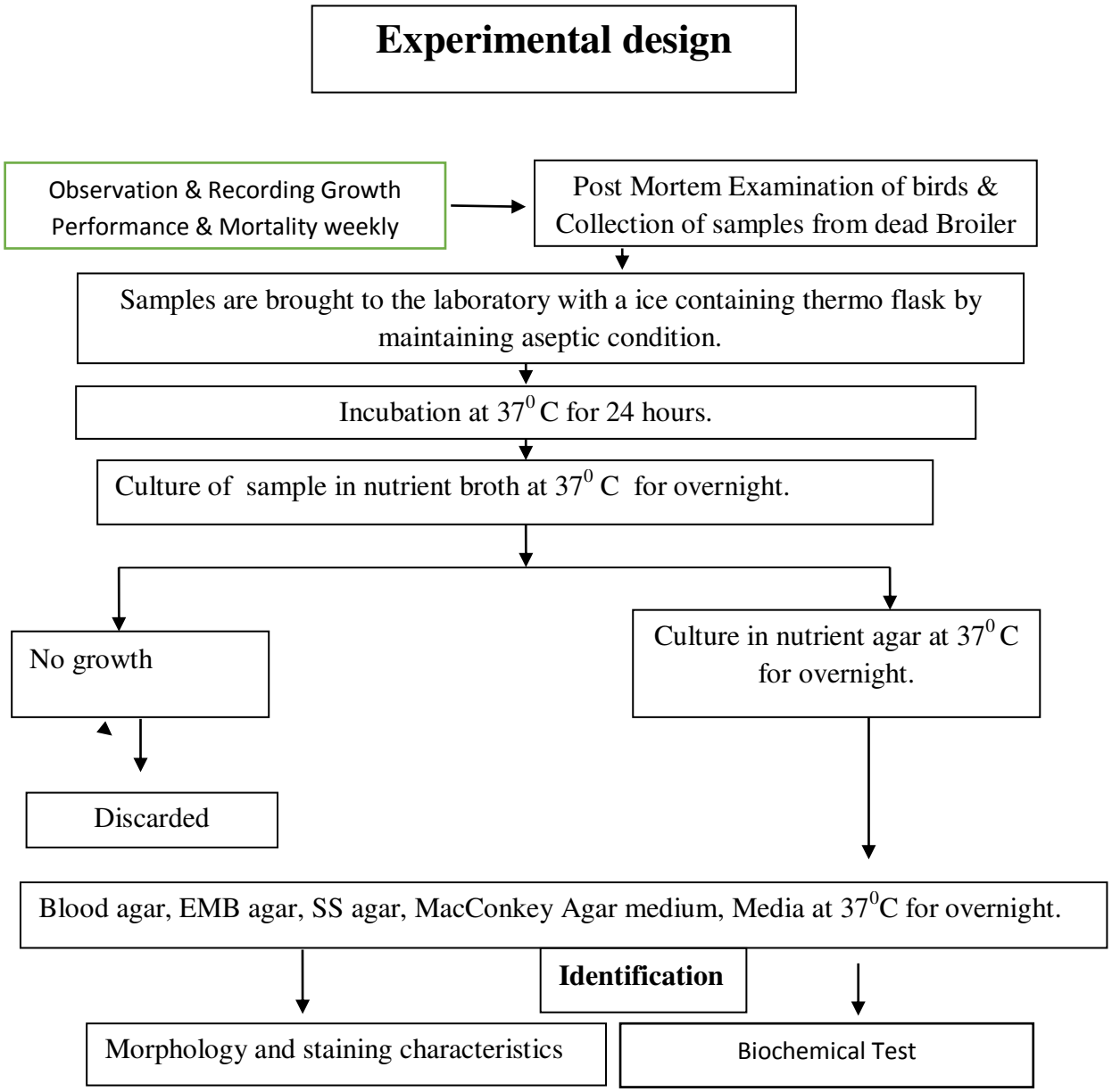



**Fig. 12: The schematic illustration of the experiments.**

### 3.2.3 Collection and transportation of samples

Samples were randomly collected directly from dead birds. The samples were carried to the bacteriological laboratory of AG Agro Industries, Gaipur, in an ice box contained ice and processed subsequently and kept it in incubator at $37^0$ C for 24 hours.

### 3.2.4 Preparation of culture media and broth

All the media, broth and reagents used in this experiment were prepared based on instruction of the manufacturer.

### 3.2.5 Nutrient broth media

13 gm of dehydrated nutrient broth (HI-MEDIA, India) was dissolved into 1 ml of distilled water.Then heated upto boiling & dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs .Sterilization was done by autoclaving at 121° C and 1.2 kg/cm2 pressure for 15 minutes. The sterility was checked by incubating at 37° C for overnight .Finally it was stored at 4° C in aerator for further use.

### 3.2.6 Nutrient agar medium

28 gm of nutrient agar powder (HI-MEDIA) was suspended in 1000 ml of cold distilled water in a flask. It was then heated to boiling. The medium was sterilized by autoclaving. It was poured into sterile Petridish and allowed to solidify. Then incubated at 37°C for overnight to check their sterility.Finally stored at 4°C in refrigerator for future use.

### 3.2.7 Blood agar medium

40gm of Blood agar base (HI-MEDIA, India) powder was suspended in 1000 ml of distilled water. After Boiling, the medium was sterilized by autoclaving at 1.2 $kg/cm^2$ pressure and 121° C for 15 minutes and 45° C. Then 5-10 % sterile defibrinated blood was added and distributed to sterile petridishes and allowed to solidify.

### 3.2.8 Eosin Methylene Blue agar

36 gm of EMB agar base (HI-MEDIA, India) was added to 1000 ml of water in a flask and boil to dissolve the medium completely. Then it was autoclaved at 1.2 $kg/cm^2$ pressure and 121° C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petridish and allowed to solidify. Then the petridishes were

incubated at 37° C for overnight to check their sterility and were used for cultural characterization or stored at 4°C in refrigerator for future use.

### 3.2.9 Mac Conkey agar medium

51.5 gm MacConkey agar base (HI- MEDIA, India) powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. Autoclaved at 1.2 kg/$cm^2$ pressure and 121° C for 15 minutes. The medium was then put into water bath at $45^0$- $50^0$C to decrease the temperature.

Then medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass Petridishes (large sized) to form thick layer. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petridishes partially removed. The sterility of the medium was checked by incubating at 37° C for overnight. The sterile medium was used for cultural characterization or stored at 4° C in refrigerator for future use.

### 3.2.10 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 adjusted to $P^H$ 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed invertedly. These were then sterilized by autoclaving at 1.2 kg /$cm^2$ 121° C for 15 minutes. The sugars used for fermentation were prepared separately 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100° C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours.

### 3.2.11 Salmonella-Shigella agar media

The SS agar plates were prepared and stored following the procedure of Cowan, (1985).An amount of 63 gm powder of SS agar base (Hi-media, India) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at $12^0$C maintaining a pressure of 15 lb pressure/sq. inch for 15 minutes. After autoclaving, the medium was put into a water bath at $45^0$C to cool down its temperature. Then 20 ml of medium was poured into each sterile petridishes and allowed to solidify. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at $37^0$C for overnight to check their sterility and then stored at $4^0$C in a refrigerator for future use.

### 3.3. Isolation and identification of microorganism.

### 3.3.1 Isolation and identification of *Escherichia coli.*

#### 3.3.1.1 Culture into different media

Streaking the lactose broth culture on EMB agar and MacConkey agar was done by using sterilized platinum loop to get isolates in pure culture. All inoculated media were incubated at 37° C for overnight .

#### 3.3.1.2 MacConkey agar

Materials from lactose fermentation tubes were inoculated into Mac Conkey agar plates.After Incubation if result is positive for *Escherichia coli,* will show rose pink color colonies.

#### 3.3.1.3 Eosin Methylene Blue (EMB)agar

Materials from lactose fermentation tubes were inoculated into EMB agar plates. After Incubation if result is positive for *Escherichia coli,* will show smooth circular colonies with dark centers and metallic sheen.

#### 3.3.1.4 'Gram's staining method

According to the methods described by Cowan and Steel (1979), Gram staining reaction was performed. If it was *Escherichia coli,* will revealed Gram negative, pink color, large rod shape appearance, arranged in single or paired.

### 3.3.1.5 Identification by biochemical test

A loopful of broth culture of the isolated were inoculated for sugar fermentation on the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol and incubated at 37°C for 18 hours. If it is positive, fermention of five sugar viz. dextrose, maltose, lactose, sucrose, and mannitol, the organisms produced acid and gas in all cases. Reddish to yellowish color in the medium indicates Acid production and appearance of gas bubble in the inverted Durham's tube indicates gas production.

### 3.3. 2 Isolation and identification of the *Salmonella* spp.

### 3.3.2.1 Culture on nutrient agar

Collected samples were directly inoculated into nutrient agar by using sterile inoculating loop and incubation is done at 37º C for 24 hours. The incubated media were then examined. Smooth, glistening and opalescent colony of bacteria were found on nutrient agar.

### 3.3.2.2 Culture in to different media

The gram negative organisms were inoculated into MacConkey agar, EMB agar and incubated at 37º C for 24 hours. The incubated media were then examined for growth of bacteria.

### 3.3.2.3 Culture on selective media

In case of EMB agar, non-metalic sheen colony was sub cultured. In case of MC agar-colorless, translucent colony was sub cultured on selective media (SS agar, BG agar, Selenite broth). In case of SS agar colorless, translucent, sometimes black colony was sub cultured. In case of BG agar, light pink colony against a rose pink background was sub cultured. Thus single pure colony was obtained. Thus single pure colony was obtained.

### 3.3.2.4 Morphological study (Gram's staining)

- At the center of a clean sterile slide , distilled water was placed with the help of sterile loop.

- With the help of bacteriological loop a Small colony was picked up and was mixed with distilled water on the slide.
- Thin smear was made on the slide.
- The smears were fixed by air drying.
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Lugol's iodine was then added to act as mordant for One minute.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- The slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to dry. The slide was examined under microscope with high power

Gram positive (violate colour) organisms are discarded and gram negative (pink coloured), small rod shaped, single or paired arranged organisms were selected ,Rahman (1995).

**3.3.2.5 Indole test**

5 ml of bacterial culture were inoculated into2 ml of peptone water and incubated for 48 hours. Kovac's reagent (0.5 ml) was added and mixed thoroughly. The tube was then allowed to stand for sometimes. The appearance of red color on the whole medium was indicated as a positive test for the production of indole by the organisms (Cheesbrough, 1985).

**3.3.2.6 MIU (Motility, Indole, Urea) medium**

Suspected colony was inoculated into the tube containing MIU medium. Incubation was done at 37°C for overnight. If there was no turbidity throughout the medium ,was indicated non-motile *Salmonella* organisms.

### 3.3.3 Isolation and identification of *Clostridium perfringens.*

#### 3.3.3.1 Culture in broth

All the suspected Clostridium organisms were inoculated into nutrient broth .Then it was kept in a candle jar .Incubation was done for 24 hours at 37°C. For maintenance of anaerobic condition 5 ml of olive oil (2-3 cm) was poured on surface of culture broth in 10 ml size test tube (Eyre, 2009).

#### 3.3.3.2 Stab culture

From 5 suspected organisms in agar containing a deep column of medium a stab culture was prepared. Then thrusted the inoculating loop to the bottom of the tube.1 or 2 cm layer of sterilized oil (Olive oil) was poured on the surface of the medium and incubated for 24 hrs at 37°C. (Eyre, 2009). Repeated passages in stab culture were performed until the culture became pure (Eyre, 2009).

#### 3.3.3.3 Gram's staining of impression smear

From jejunum of intestine impression smears from 5 samples prepared. These were stained with Gram's staining method. The stained slides were examined according to the procedure described by Rahman (1995). Among these 1 samples revealed the presence of Clostridium organisms.

#### 3.3.3.4 Carbohydrate fermentation test

This was performed by inoculating 5 ml of nutrient broth culture of the organisms into the tubes containing different sugar media and incubated for 72 hours at 37°C (Eyre, 2009).

#### 3.3.3.5 Methyl red (MR) test

Colonies of each isolate ,from 5 samples were incubated at 37°C & After that 2 - 4 drops of methyl red solution were added to the test tube which was incubated for 5days for MR test (Eyre, 2009).

#### 3.3.3.6 Voges-Proskauer (V-P) test

Colonies of each isolate ,from 5 samples was performed by adding 6 ml of VP reagent-1 and 0.2 ml of VP reagent-2 for each ml of culture. The ingredients were mixed thoroughly and allowed to stand for 2 minutes (Eyre, 2009).

#### 3.3.3.7 Indole test

Colonies of each isolate ,from 5 samples were tested for indole positivity. 2 ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours at 37°C. 0.5 ml Kovac's reagent was added, shaken well and examined after 1 minute (Eyre, 2009).

#### 3.3.3.8 Blood agar for hemolytic activity

Colonies of each isolate ,from 5 samples were Spread into Blood agar media (Sheep Blood) from stab culture and were incubated anaerobically for 24 hours at 37°C (Eyre, 2009).

#### 3.3.3.9 TSI agar slant reaction

Colonies of each isolate ,from 5 samples were inoculated into TSI agar slants by using stab or streak method (Eyre, 2009).

# CHAPTER 4

# RESULTS

# CHAPTER 4

# RESULTS

## 4.1. Mean body weight record

The effect of administration probiotics & prebiotics were evaluated in chickens against control group (Table 3, 4, 5 & 6). The mean initial weight of chicks for all groups were almost similar, which was 39 g and above as recorded on day 1. Significant increase of body weight was recorded in probiotic administrated group A, prebiotic administrated group shown the increase was seen in Group B, then group C chicken for control group. Concerning the mean body weight recorded as compared to healthy control (Group C) on day 30, Group A (1772.7 ±56.89) chickens showed highest performance, followed by Group B (1625.1 ±35.80). Chickens belonging to Group C showed lowest performance in terms of body weight (1394.1 ±62.60).


**Control** **Prebiotics** **Probiotics**

**Fig 13: Body weight Recording based on group (Final Weight)**

**Table 3: Effects treatments on broilers Mortality**

| Treatment | 1st Week | 2nd Week | 3rd Week | Day 30 | Total Mortality (%) |
|---|---|---|---|---|---|
| Saltose Plus | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 % |
| Agrimos | 0.00 | 2 pcs (1.33%) | 0.00 | 0.00 | 1.33% |
| Control | 13.00 | 11.00 | 6.00 | 0.00 | 20 % |

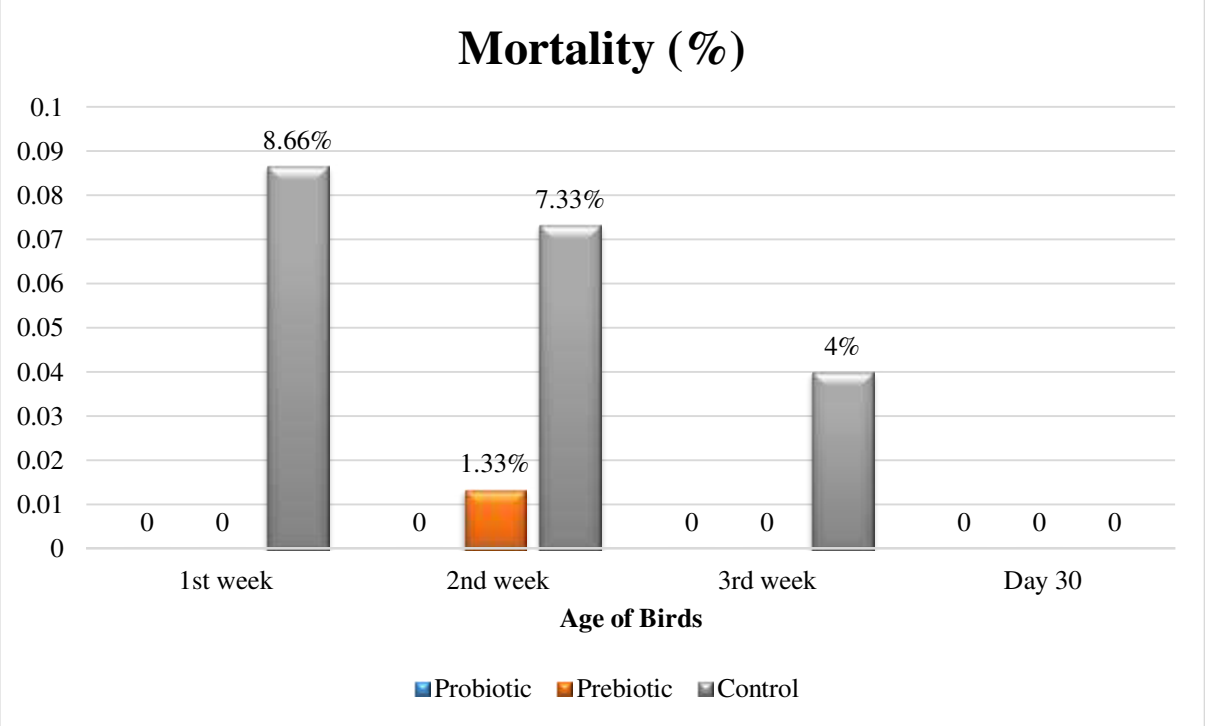


**Table 4: Effects treatments on broilers daily feed intake (DFI)**

| Treatment | 1st Week | 2nd Week | 3rd Week | Day 30 | Average |
|---|---|---|---|---|---|
| Saltose Plus | 24.67±3.06 | 53.82±9.59 | 96.79±6.19 | 171.66±18.47 | 5.25±1.26 |
| Agrimos | 23.85±8.84 | 53.57±11.35 | 92.85±6.72 | 173.24±20.62 | 5.33±2.42 (NS) |
| Control | 24.16±5.35 | 53.21±5.66 | 96.18±8.10 | 161.94±24.53 | 4.92±1.30 |
| **P Value** | 0.630 | 0.298 | 0.000 | 0.000 | 0.000 |

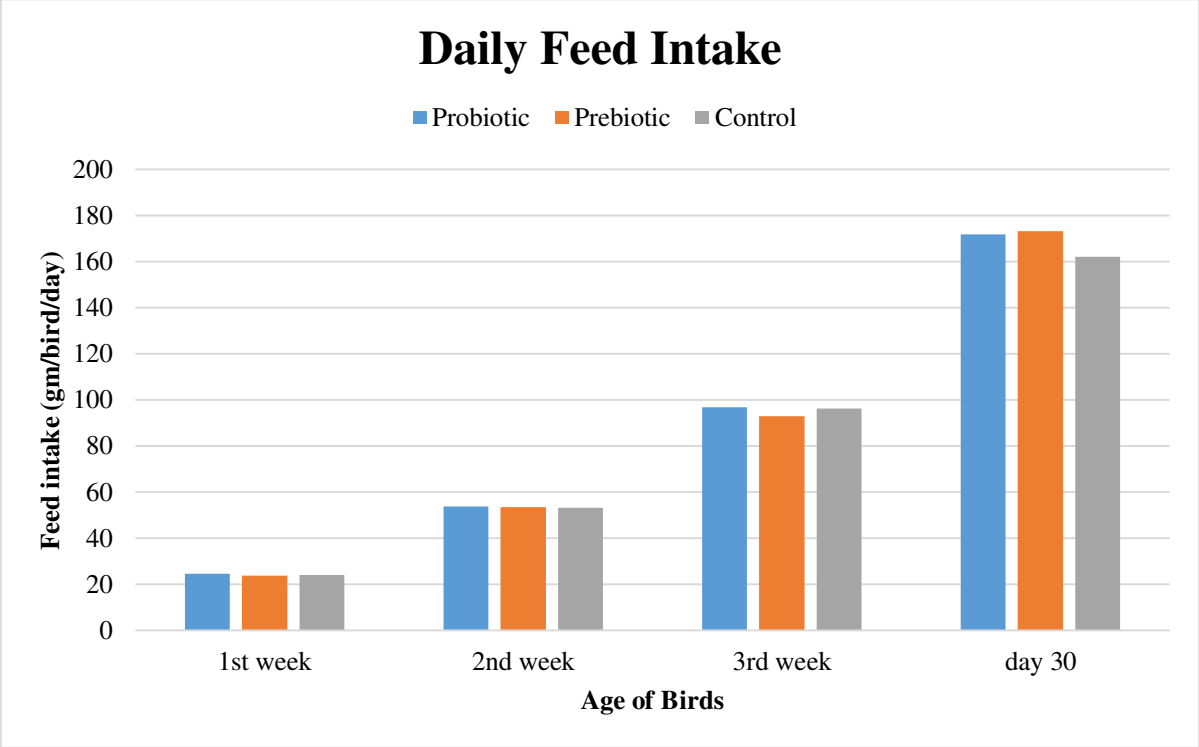


**Table 5: Effects treatments on Average Body Weight gain in gm (AWG)**

| Treatment | 1st Week | 2nd Week | 3rd Week | Day 30 | Average |
|---|---|---|---|---|---|
| Saltose Plus | 196.13±15.67 | 489.52 ±18.49 | 968.56 ±19.37 | 1772.7 ±56.89 | 56.30±4.55 |
| Agrimos | 185.56±13.76 | 472.19 ±34.01 | 951.98±51.89 | 1625.1 ±35.80 | 51.41±3.35 |
| Control | 141.23±12.19 | 440.67 ±33.84 | 891.47 ±41.13 | 1394.1 ±62.60 | 44.74±5.35 |

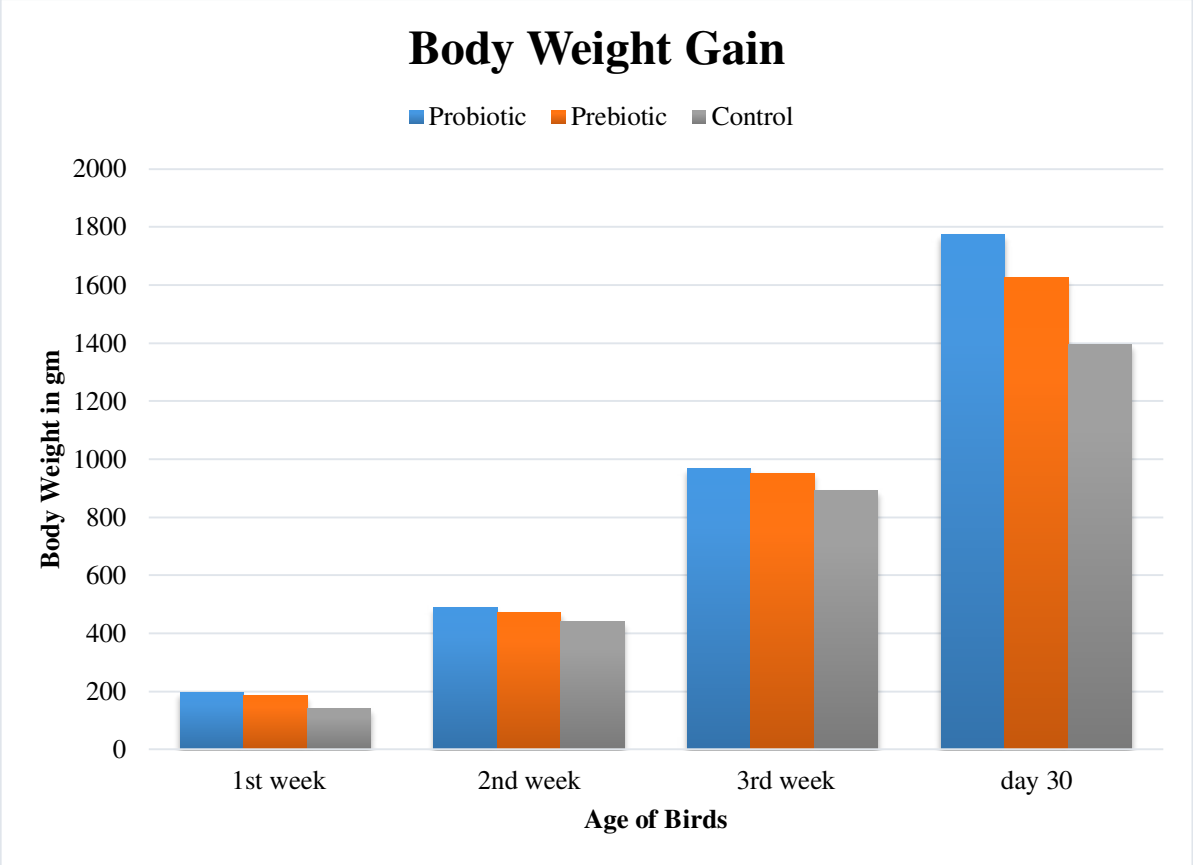
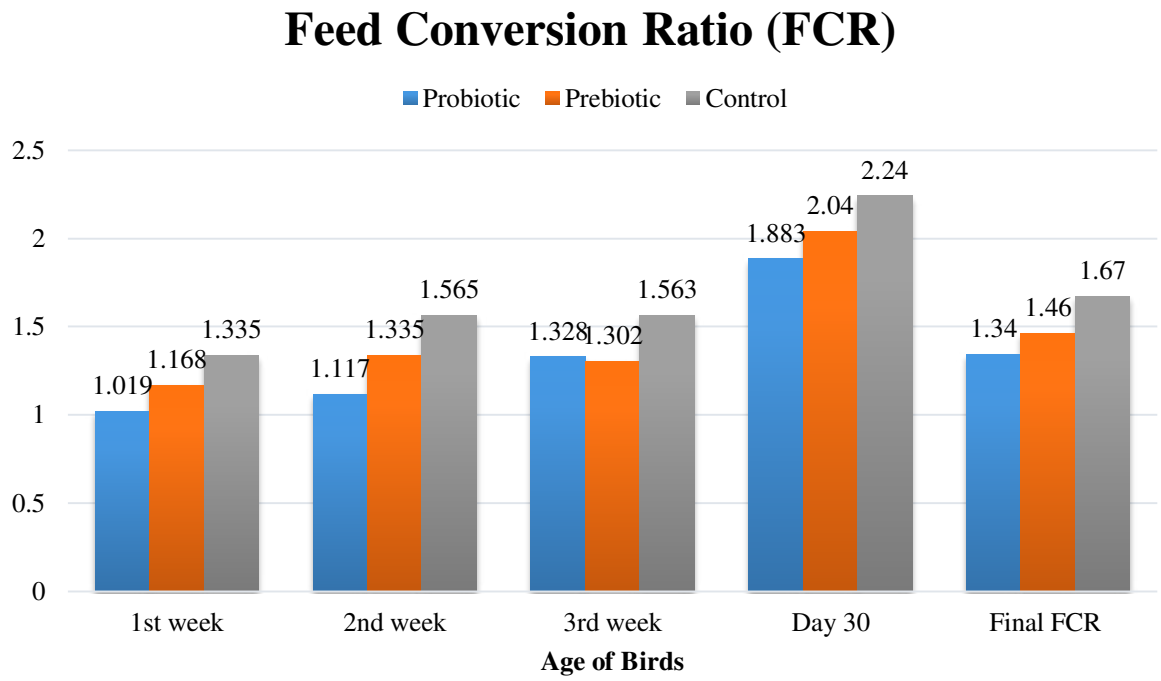


**Table 6: Effects treatments on feed conversion ratio (FCR)**

| Treatment | 1st Week | 2nd Week | 3rd Week | Day 30 | Average | |
|---|---|---|---|---|---|---|
| Saltose Plus | 1.019±0.06 | 1.117±0.27 | 1.328±0.26 | 1.883±0.45 | 1.34 | 0.02±0.007 |
| Agrimos | 1.168±0.40 | 1.335±0.22 | 1.302±0.11 | 2.040±0.38 | 1.46 | 0.03±0.01 |
| Control | 1.335±0.28 | 1.565±0.16 | 1.563±0.35 | 2.240±0.41 | 1.67 | 0.03±0.002 |
| **P Value** | 0.792 | 0.000 | 0.001 | 0.002 | 0.000 | |



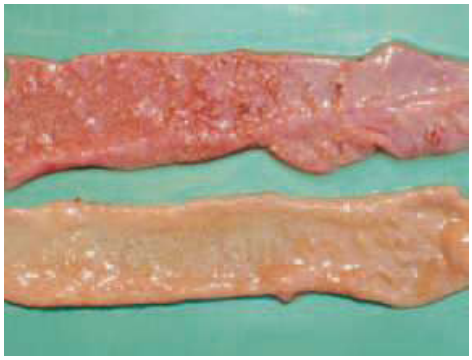
### 4.2. Post-mortem findings of Dead birds

**Group B**

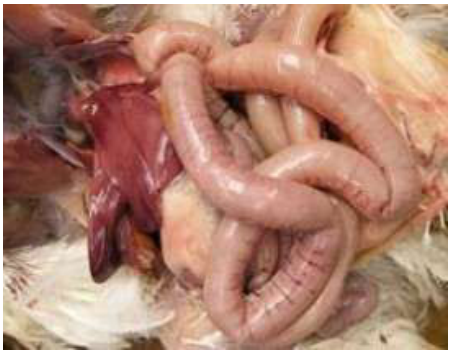
In Group I the gastrointestinal tract appeared healthy. The caeca did not reveal any change.

**Group C**

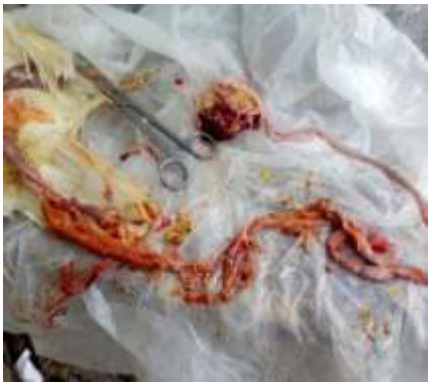
- Balloning of intestine, Distended intestine filled with foul smelling Brown fluid & ulceration of intestinal lining. About 13 birds shown near about same lesions.



Ulceration in intestine



Distended Intestine



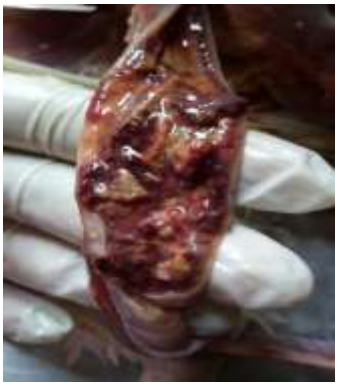


**Fig 14: Post mortem findings indicating Necrotic enteritis**

- Unabsorbed yolk at 7 days & 11days old dead birds, found in 7 birds.

Day 7 Day 11

**Fig 14: Post mortem findings indicating Colibacillosis.**

- Greenish discoloration of liver found in dead birds found at day 6 to day 10 old birds

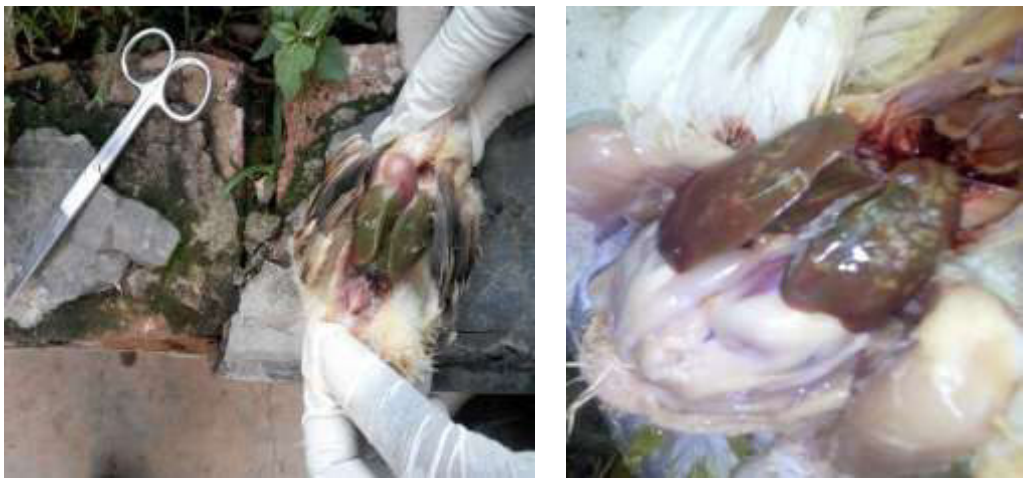


**Fig 15: Post mortem findings indicating Salmonellosis**

Result of morphological, staining, cultural, biochemical characteristics of isolated bacteria from 32 dead birds are presented in the table and described below under the following headings:

### 4.3 Diagnosis of Enteric Bacteria

Thirty two selected samples of dead birds showed different staining characters in impression smears (Table 7).

**Table 7: The prevalence of bacteria from 32 dead broiler chickens**

| Isolated Organism | Number of birds | Percentage (%) |
|---|---|---|
| *Clostridium* sp. | 21 | 65.65 |
| Mixed bacteria *(E. coli, Salmonella spp.)* | 8 | 25.00 |
| Unidentified bacteria | 3 | 9.35 |

### 4.3.1 Isolaton and identification of *Escherichia coli*

All of the 8 selected sample was shown positivity for *E. coli* infection.

**Table 8: Cultural, morphological and biochemical properties of isolated Escherichia coli**

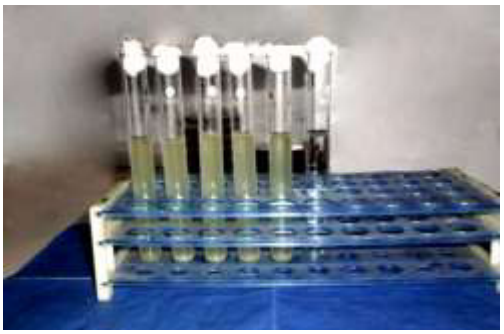
| Cultural characteristics | | Biochemical Characteristics | | Staining and morphological Characteristics |
|---|---|---|---|---|
| EMB Agar | MC Agar | Tests | Results | Staining properties |
| Smooth, circular, black color colonies with metallic sheen were produced | Bright pink color, smooth colonies were produced smooth colonies were | Dextrose | AG | Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain |
| | | Maltose | AG | |
| | | Lactose | AG | |
| | | Sucrose | AG | |
| | | Mannitole | AG | |
| | | Catalase test | + | |
| | | Indole | + | |
| | | MR | + | |
| | | MIU | - | |
| | | TSI | + | |
| | | VP | - | |

**Legends :**

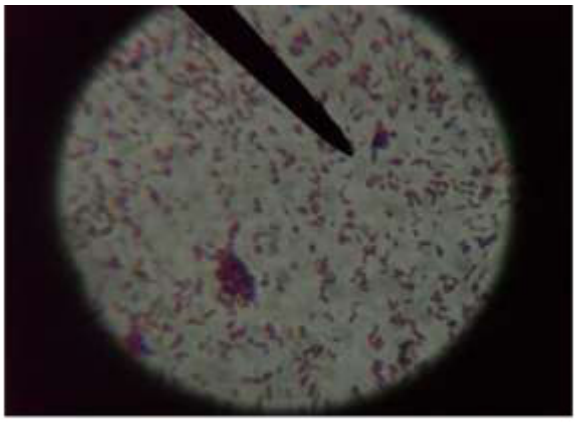
AG = Acid and Gas, MR = Methyl-Red test, VP = Voges-Proskauer test,

+ = Positive reaction, - = Negative reaction, EMB = Eosin Methyle Blue, MC = MacConkey

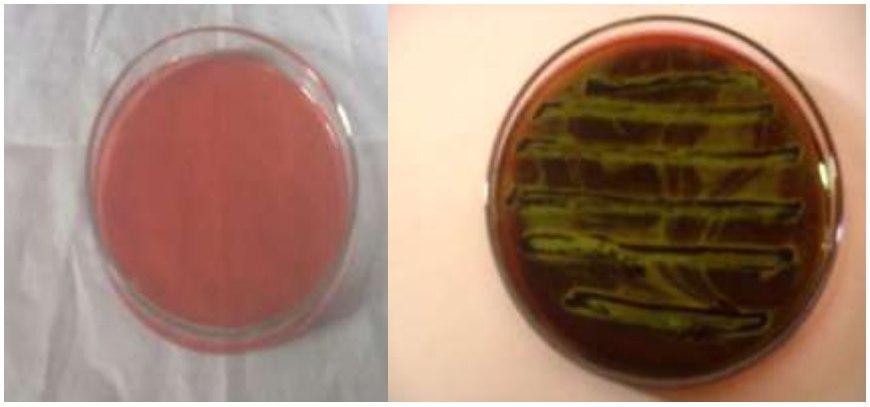
Thick grayish white colony of E. coli on Nutrient agar.



Culture of E. coli in nutrient broth showing turbidity (left) and uninoculated contro (right).



Gram-negative single or paired short plump rods of *E. coli*



Metallic sheen colony of E. coli on **EMB** agar(right)and uninoculated control (left)

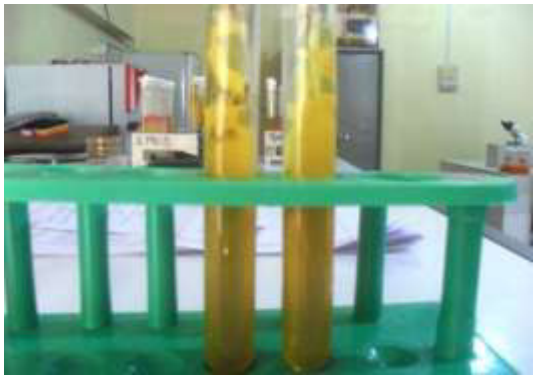
**Catalase(positive)** for*E. coli* with gas bubble formation.

**Indole test** showing positive results with a red color in the reagent layer indicating indole production with reaction of *E. coli* (right) and uninoculated control (left).

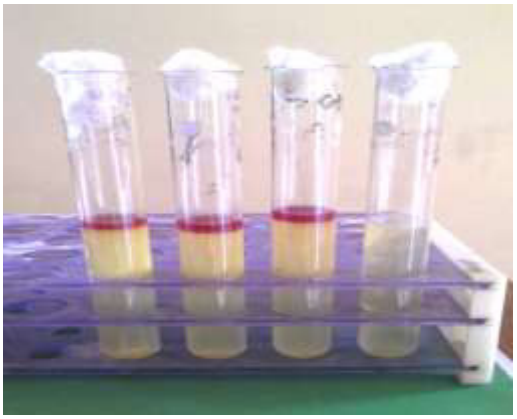
Yellowish green colour colony produced of *E. coli* on **Brilliant Agar media**(right) and uninoculated control (left).

*E. coli* are grown on Simmons citrate agar media(left) , the medium is changed to blue colour

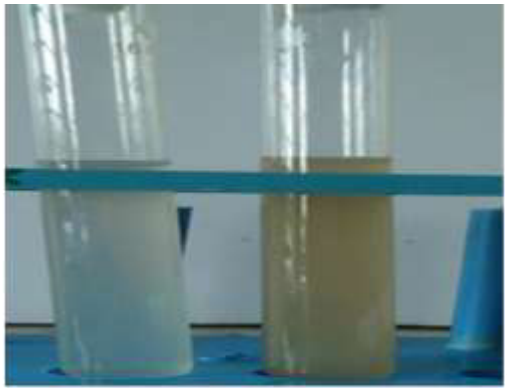
*E. coli* produces dark pink color colony in Mac Conkey agar (right)and uninoculated control (left)



Culture in **Triple Sugar Iron (TSI)** agar slant reaction showing yellow slant and yellow butt(left) and production of gas by *E. coli*.

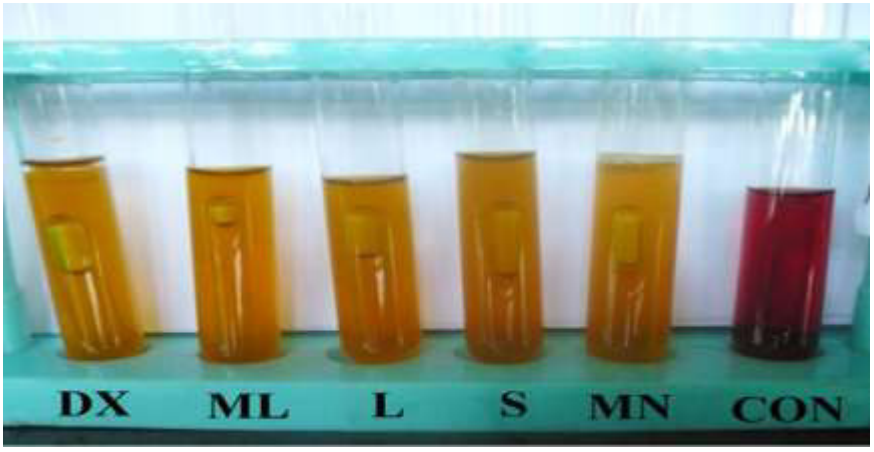


**Methyl-Red test** for *E. Coli* showing bright red color (right) and uninoculated control (left)



**Voges-proskauer test** for **E. coli.** Showing no change of the medium (right) and uninoculated control. (night).

**MotilityIndole Urease test** causing **turbidity** Urease production with indole positive by *E. Coli* (left) and uninoculated control (right).




Fermentative activity of *Escherichia coli.***with five basic sugars** (DX=Dextrose, ML= Maltose, L= lactose, S= Sucrose, M= Mannitol, C= Control) with production of acid and gas. Change of medium color reddish to yellowish and gas bubble formation.

**Fig 16: Microbiological test for Collibacillosis**

### 4.3.2 Isolation and identification of *Salmonellae spp.*

All of the 8 selected sample was shown positivity for *E. coli* infection.

#### 4.3.2.1 Cultural characteristics of *Salmonellae spp.*

**Table 9: Cultural characteristics of *Salmonella spp.* in different culture media**

| Media used | Colony characteristics | Isolated organism |
|---|---|---|
| Salmonella-Shigella agar | Translucent, Smooth, Small round black centered colonies | *Salmonella* spp. |
| MacConkey agar | Colorless, smooth, transparent, raised colonies | |
| Brilliant green agar | Pale pink color colonies against a yellowish background. | |
| TSI agar | Transparent, smooth round colonies | |
| Nutrient agar | Translucent, opaque, smooth colonies | |
| Nutrient broth | Turbidity in the broth | |

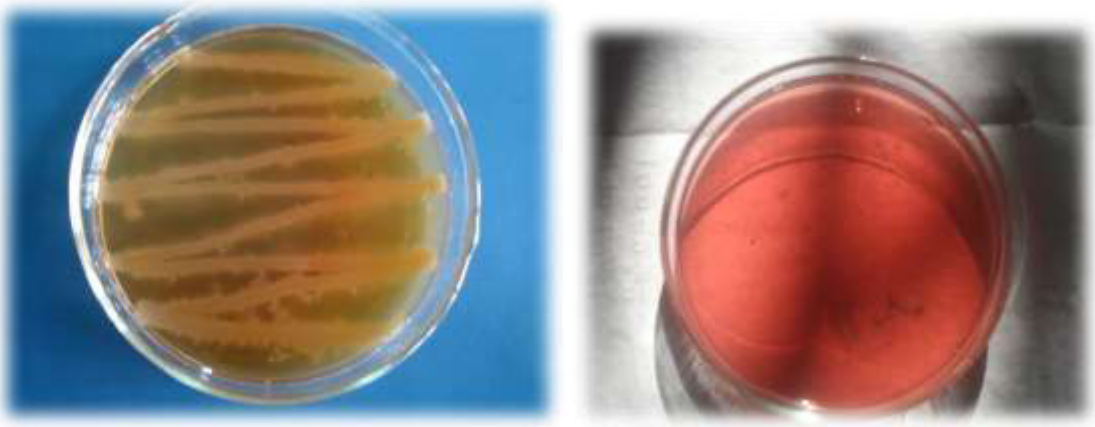
#### 4.3.2. 2 Results of motility test

Isolates was found to be non motile when examined using hanging drop slide under microscope. The Characteristics of chicken *Salmonellae* isolates by Gram's staining method and motility test are presented in Table 5.

#### 4.3.2.3. Results of biochemical test

**Table 10: Results of biochemical test of *Salmonella spp.***

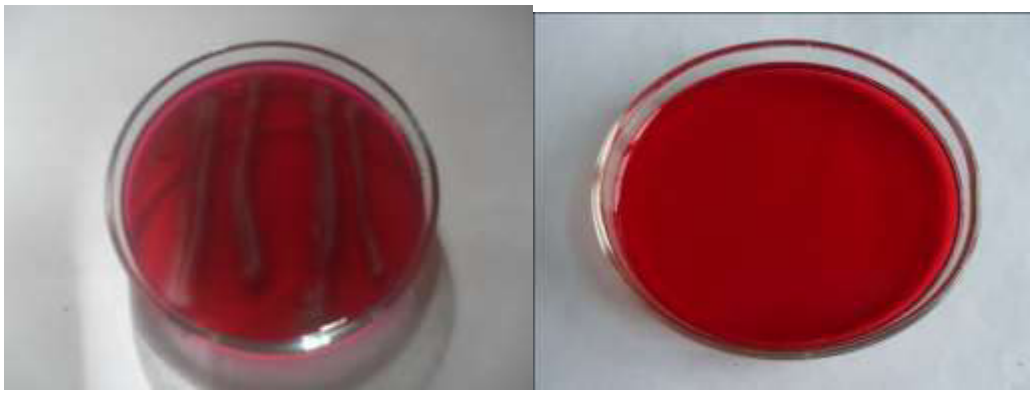
| Biochemical Characteristics | | Staining and morphological Characteristics |
|---|---|---|
| Tests | Results | Staining properties |
| Dextrose | AG | Gram-negative, pink colored, small rod shaped appearance, arranged in single or paired under the microscopic examination. |
| Maltose | AG | |
| Lactose | - | |
| Sucrose | - | |
| Mannitole | AG | |
| Indole | - | |
| MR | + | |
| MIU | + | |
| VP | - | |



Growth of *Salmonella in* MacConkey agar showing colorless, smooth, pale and transparent raised colonies (left) and control (right).

Growth of *Salmonella* in EMB agar showing pink color colonies (left) and control (right).

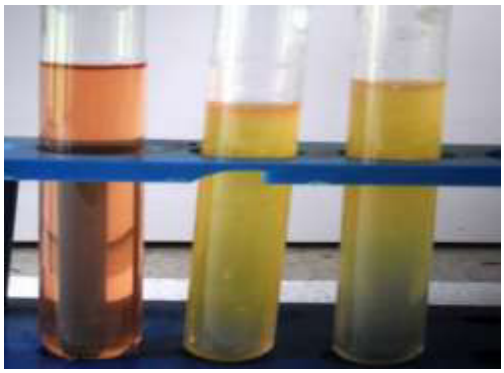
Black center colonies produced by *Salmonella* on SS agar(left) and un-inoculated control (right).



Growth of *Salmonella* in Brilliant green agar showing pale pink color colonies against a pinkish background (left) and control (right).

Culture of *Salmonella* on Simmons citrate agar showing deep blue coloration of the medium(left) and Control(right).

Indole test showing no indole ring in the medium(right) and control (left)
**Indole test(*Salmonella).***



MIU test showing absence of turbidity indicating non motile *Salmonella spp*(Right) and control(left)

**Fig 17: Microbiological test for Salmonellosis**

### 4.3.3 Isolation and identification of *Clostridium perfringens.*

#### 4.3.3.1 Cultural characteristics of *Clostridium perfringens*

Twenty one isolates that were tentatively identified as *Clostridium sp.* were used for culture.

**Table 11: Shows the cultural characteristics of *Clostridium perfringens***

| Media used | Characteristics | Isolated organism |
|---|---|---|
| Stab culture | Tubular cylindrical colonies | *Clostridium perfringens.* |
| Blood agar | Round, smooth, circular, gray-white color and surrounded by a typical zone of haemolysis (β-haemolysis). | |
| TSI agar | The slant was turned yellow ,$H_2S$ was also produced giving a black precipitate to the upper layer of the slant. | |

#### 4.3.3.2 Carbohydrate fermentation test & Biochemical test result

All of the 21 selected sample was used for carbohydrate fermentation & biochemical test.Result were noted in table 8. Only Sample no. 06, 11,12,17,18 were shown positive result for clostridial organism.

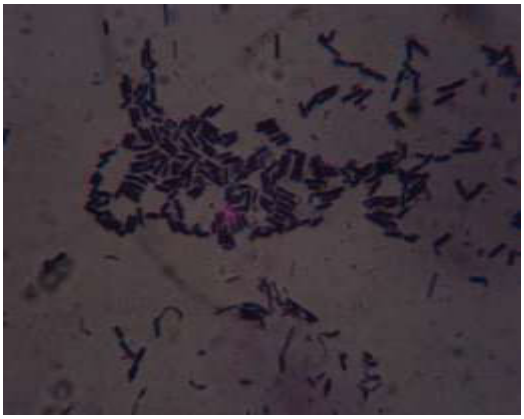
**Table 12: Shows carbohydrate fermentation test result and biochemical test result of *Clostridium perfringens*.**

| Sample No. | Dex | Lac | Suc | Mal | Man | Ind | MR | VP | Identified Organism |
|---|---|---|---|---|---|---|---|---|---|
| Sample 06 | AG | AG | AG | AG | - | - | - | - | *Clostridium perfringens* |
| Sample 11 | AG | AG | AG | AG | - | - | - | - | *Clostridium perfringens* |
| Sample 12 | AG | AG | AG | AG | - | - | - | - | *Clostridium perfringens* |
| Sample 17 | AG | AG | AG | AG | - | - | + | - | *Clostridium perfringens* |
| Sample 18 | AG | AG | AG | AG | - | - | - | - | *Clostridium perfringens* |

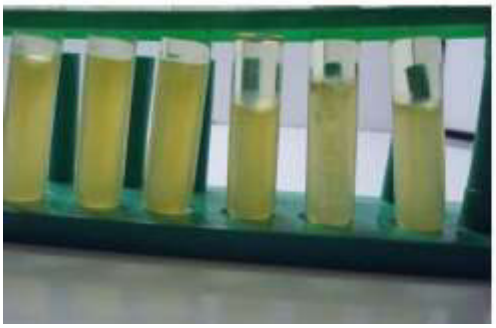
**Legends:**

Dex = Dextrose, Lac = Lactose, Suc = Sucrose, Mal = Maltose, Man = Mannitol, Ind = Indole, MR = Methyl Red, VP = Voges Proskauer, A = Acid, AG = Acid and Gas

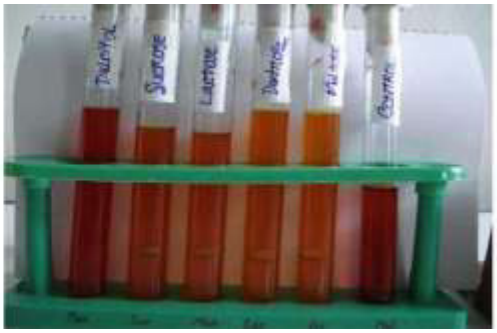
TSI slant agar shown black color colony



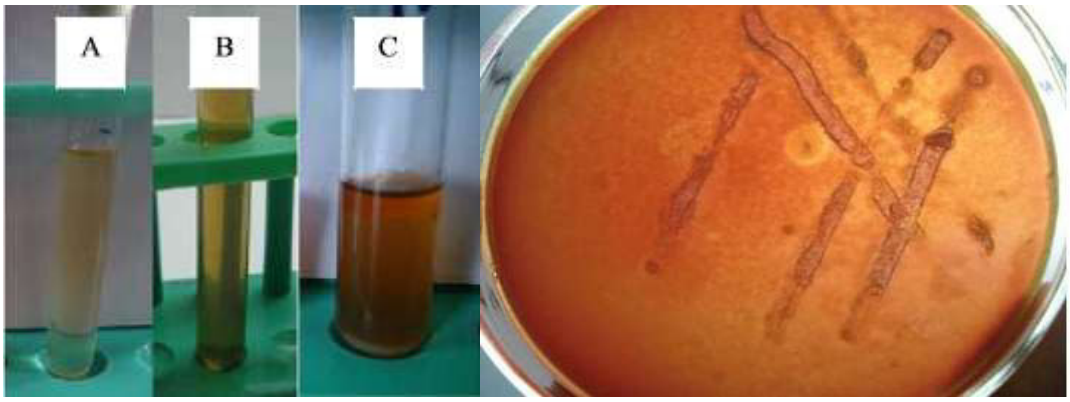
large, thick, Rod shape,Gm +, bacilli



Culture of *Clostrdium* shows tubular cylindrical colonies in stab culture.



Carbohydrate fermentation test produces acid and gas




A, B& C: Shows M-R, V-P & indole test negative

Culture of Clostridium shows a typical zone of haemolysis (β-haemolysis)

**Fig 18: Microbiological test for Necrotic Enteritis**

# CHAPTER 5

# DISCUSSION

# CHAPTER 5

# DISCUSSION

Through genetic improvements, the productivity of broilers has improved significantly. While this is a good thing for the poultry industry, increased rearing density has concentrated and increased disease challenges making birds more susceptible to various pathogens especially enteropathic microbes such as *E. coli*, Salmonella spp. & *Clostridium perfringens*.

This increased susceptibility has resulted in the use of antimicrobial growth promoters which are primarily used to enhance gut health and control sub-clinical challenges. There has long been interest in finding alternatives to antibiotics for poultry production. Resident microbes in the birds' digestive tract have a profound effect on some of the physiological processes of their host. With this in mind, it is important to understand the dynamics of the intestinal microbial ecology of the chicken to find alternatives to antibiotics. Under normal circumstances there is a delicate balance of beneficial and pathogenic bacteria in the gastrointestinal tract (GIT). This is influenced by symbiotic and competitive interactions and relationships. The microbial communities will not only protect the GIT but also enhance productivity in the host.

The use of probiotics and prebiotics are two approaches that have been examined and can potentially reduce enteric diseases in poultry and also enhance their productivity. These substances have been proposed to assist in the prevention of carcass contamination and improve the immune response in the chicken (Huang et al., 2004). Probiotic and prebiotic foods are by no means a novel approach; in fact, they have been consumed for centuries either as natural components of foods or as fermented foods, e.g. yogurt.

Probiotics have been defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance' (Fuller, 1989). The probiotic mode of action is by 'competitive exclusion', meaning there is competition for attachment sites in the GIT. The bacteria of the probiotic attach to the intestinal mucosa, thereby forming a physical barrier that blocks the attachment of pathogenic bacteria (Furlan, 2005). They also produce antibacterial compounds and enzymes and stimulate the immune system.

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). The most common prebiotics are oligosaccharides, which are non-digestible carbohydrates. The way in which prebiotics act is by (1) supplying nutrients to beneficial microbes, or (2) tricking pathogenic bacteria into attaching to the oligosaccharide rather than to the intestinal mucosa. This reduces the intestinal colonization thereby decreasing the incidence of infection in the birds. Because the oligosaccharide is non-digestible, the microbes that are attached will travel along the GIT with the ingesta, and are excreted from the bird along with other undigested food.

Enteric diseases are of major economic concern in the poultry industry. They result in lost productivity, increased mortality in flocks and also potential contamination of poultry products, which leads to human food safety concerns. The use of antimicrobials in poultry feed has been curtailed due to concerns of bacterial antibiotic resistance. Alternatives to the use of antibiotics in poultry feed should be aggressively evaluated under field conditions. Probiotics and prebiotics are good alternative candidates. The present research work was undertaken to know the potentiality of prebiotic & probiotic on broiler & identify the causal agent of necrotic enteritis, which has been hindering the poultry development in Bangladesh and causing economic losses. In the present study, the results also shown that the treatments had significant effects in ADG and FCR. However, the birds fed with Probiotic, resulted in the most favorable ADG while broilers fed with ratios of prebiotic were ranked second and broilers in control group were ranked Third. Feed conversion ratios are also provided in Table 6. The results showed that the ratio which includes probiotics was associated with the best outcome. The birds which were feed with saltose plus have the lowest FCR (1.34) than the broilers fed with agrimos (1.46) and basal diet (1.67).The results of current study are in line with other studies that investigated effect of probiotics on chicken broilers. The primary role of a diet is to provide enough nutrients to fulfill metabolic requirements of the body and also to modulate different functions of the body. Probiotics are beneficial microorganisms that can be suitably used to improve growth performance and health of broiler chickens. Ignatova et al. conducted a research to evaluate effects of dietary inclusion of probiotics on chicken's performance. Two hundred 1-day old male White Plymouth Rock-mini chickens were studied for this research purpose. However, results revealed that probiotic

supplementation has positive effects on final body weight by 14.4% , increased feed intake by 7.7%, and improved feed utilization by 8.1%. Several studies have been conducted to evaluate the ability of probiotics to change the type and number of the microflora in the digestive tract and results show that dietary supplementation of probiotic have a positive effect on growth performance and would significantly increase ADG and FCR in broiler chicks receiving probiotics .The results of current study also show that feeding broilers with probiotics had no significant effect on FI, but improved them as numeral and increasing FI itself could improve growth performance. From the records of mortality and recovery rate ,it showed that among the groups of the probiotic trial the Group A gave no mortality rate, Group B had 1.33% & Group C had the highest (20%) mortality.

Three different bacteria (*E. coli*, *Salmonella* spp., *& Clostridium perfringens*.) were isolated from the internal organs of broiler. This is in line of findings by Malmuthuge *et al.* (2012) and Voidarou *et al.* (2011). Considering all the 32 samples, *E. coli* was isolated from 8 (25%) samples. This finding is consistent with that of Awad- Alla *et al*. (2010) and Aguirre *et al*. (1992) who described a prevalence of 51% in broiler and 52% in black-billed ducks, respectively. The prevalence of *Salmonella spp.* was 8% in broiler, which is supported by Cardinale *et al*. (2003), Temelli *et al.* (2012) and Alcaine *et al.* (2007). However, a significant variation regarding prevalence of *Salmonella* spp. was described in other findings, such as 17.9% by Tibaijula *et al*. (2003), 14.37% by Petrovic *et al.* (2011) and 13% by Ellerbroek *et al* . (2010). Similarly, Afroz et al. (2012) reported a 26.02% prevalence of *Salmonella* spp. in internal organs of layer. These variations might be due to difference in sample size, geographical location and type of bird. Routine methods of bacterial cultures in different media, specific colony characters, microscopic examination, different staining techniques and different types of biochemical tests were used for the isolation and identification of Clostridium perfringens. The collected 21 sample in nutrient broth were kept in a candle jar and incubated for 24 hours at 37°C. For maintenance of anaerobic condition olive oil (2-3 cm) were poured on surface of culture broth in test tube and this technique was reported by Eyre (2009). In Gram's staining, the morphology of the isolated bacteria was Gram-positive, rod-shaped, anaerobic, spore-forming single or paired in arrangement which was supported by several authors (Rhodehamel et al., 1998 and Shamimuzzaman, 1999). The overall prevalence of C. Perfringens, 65.65% is high when compared to similar

works done in other places (Agarwal et al., 2000; Bachhil and Jaiswal, 1989; Singh et al., 2005; Elham and Nahla, 2011). The higher prevalence in the present study could be attributed to the heat shock in the medium during isolation in order to kill non-spore forming aerobic bacteria. This is in agreement with the work by Qiyi and McClane (2004) who reported C. perfringens at a rate of 2% and 29% in heat shocked and non-heat shocked samples. Clostridium perfringens isolates were detected in approximately 1.4% of 900 surveyed non-outbreak American retail Endale food (Qiyi and McClane, 2004). It was suggested that vegetative cells were killed by heat shocking. This is contrary to the findings of Endale et al. (2013), who isolated C. perfringens from another other than meat samples.

Additional research is required for further evaluation of efficacy of probiotic & prebiotic with characterization of the bacterial isolates described in this study.

# CHAPTER 6

# SUMMARY

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A total of 450 broiler birds were used for this study. Birds will be weighed (100%) at day old, the end of starter, grower periods for each brand of feeds, and feed consumed for each type of feed to be recorded accurately. The feeds used for this trial was pretreated with Probiotics (Saltose Plus-Opsonin Pharma Limited & Preboitics (Agrimos-Lallemand Animal Nutrition). Samples from dead chicken were collected during the period from July to September 2017. The laboratory works were conducted in the Microbiological Laboratory of AG Agro Industries, Chandona Chourasta, Gazipur, Bangladesh. A series of bacteriological methods were used for the isolation and identification of different types of enteric bacteria. Different types of ordinary, enriched and selective media such as Nutrient broth, Nutrient agar, Mannitol Salt agar, Blood agar, Brilliant Agar, Eosine Methylene agar, Cook Meat Media test, MacConkey Agar, were used for the determination of the cultural characteristics of the different types of isolated bacteria. Biochemical properties of the isolated bacteria were studied by sugar fermentation, indole test, catalase TSI test, and MR-VP test. On the basis of morphology, staining, cultural and biochemical characteristics, the isolated organisms were identified as, *Escherichia coli, Salmonella spp. & Clostridium perfringens.* Bacteriological examination of total samples 32 from dead birds. Out of 32 samples, 8 were *Escherichia coli (25%)& Salmonella spp.* , 21 were *Clostridium perfringens* (65.65% ), and 3 were other spp(9.35%). The body weight (1772.7±56.89) was significantly increased in the treatment groups fed with probiotics diet from 1$^{st}$ days to 30$^{th}$ days old, then the birds fed with prebiotic (1625±35.80). Weight (1394±62.60) was also significantly decreased among the control groups. The birds which were feed with saltose plus have the lowest FCR (1.34) than the broilers fed with agrimos (1.46) and basal diet (1.67).

From the results of the present study, it may be recommended that:

1. Efficacy of probiotic is much stronger than prebiotic but both of these are very helpful for prevention of enteric disease & enhancing the growth of broiler chicken.
2. Probiotic or Prebiotic singly or combinely can use as an alternatives to antibiotic.
3. *Escherichia coli, Salmonella spp, Clostridium perfringens* were the major enteric disease forming causal agents found in of the broiler chicken.

# CHAPTER 7

# CONCLUSION

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Poultry meat is one of the most important sources of animal protein in the world today. As the world population continue to increase so is the demand for poultry meat. Infections caused by pathogenic microorganisms such as Eimeria, Salmonella, Clostridium etc continue to threaten the poultry industry. Such infections are responsible for reduced growth rates and consequent economic losses in poultry. Traditionally, antibiotics growth promoters are used to treat infected chickens. Unfortunately, the long term and extensive use of antibiotics for veterinary purpose may eventually result in selection for the survival of resistant microbial species, thereby posing a threat to both animal and human health. Consequently, some countries have restricted the use of AGP in poultry. In this study I propose the use of feed supplements containing probiotics, prebiotics and enzymes as alternative to conventional AGP. This study suggests that, probiotic might be used as an alternative and safe nutritional and dietary supplement in broiler production.

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# APPENDICES

# APPENDICES

**Composition of Media**

**1. Selenite broth**

| **Ingredients** | **g/L** |
|---|---|
| Casein enzymic hydrolysate | 5.0 |
| Lactose | 4.0 |
| Sodium phosphate | 10.0 |
| Final pH(at 25°C) | 7.0±0.2 |

**2. Nutrient broth**

| **Ingredients** | **g/L** |
|---|---|
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| Final pH(at25°C) | 7.4±0.2 |

**3. Nutrient agar**

| **Ingredients** | **g/L** |
|---|---|
| Beef extract | 3.0 |
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Agar | 20.0 |
| Final pH | 7.1±0.1 |

**4. Salmonella Shigella agar**

| **Ingredients** | 5.00 gm |
|---|---|
| Peptic digest of animal tissue | |
| Beef extract | 5.00 gm |
| lactose | 10.00 gm |
| Bile salts mixture | 8.50 gm |
| Sodium citrate | 10.00 gm |
| Sodium thiosulphate | 8.50 gm |
| Ferric citrate | 1.00 gm |
| Brilliant green | 0.00033 gm |
| Neutral red | 0.025 gm |

| | | |
|---|---|---|
| | Agar | 15.00 gm |
| | Distilled water | 1000 ml |
| | Final pH(at25°C) | 7.0±0.2 gm |
| **5.** | **Brilliant green agar** | |
| | **Ingredients** | gtL |
| | Lab- Lemco power | 5.0 |
| | Bacteriological pepton | 10.0 |
| | Yeast extract | 3.0 |
| | Disodium hydrogen phosphate | 1.0 |
| | Lactose | 10.0 |
| | Sucrose | 10.0 |
| | Phenol red | 0.09 |
| | Brilliant areen | 0.007 |
| | Agar | 12.0 |
| | Final pH~-O? | 6.9±0.2 |
| **6.** | **MacConkey Agar** | |
| | **Ingredients** | **g/L** |
| | peptone | 17.0 |
| | Protease peptone | 3.0 |
| | Lactose | 10 |
| | Bile salt | 1.5 |
| | Sodium cholride | 5.0 |
| | Agar | 13.5 |
| | Neutral Red | 0.03 |
| | Crystal violet | 0.001 |
| | Final pH | 7.1±0.2 |
| **7.** | **Eosine methylene blue agar** | |
| | **Ingredients** | **g/L** |
| | Peptone | 100 |
| | Lactose | 10.0 |
| | K2HP04 | 2.0 |
| | Eosin | 0.4 |
| | Methylene blue | 0.065 |

| | | |
|---|---|---|
| | Agar | 20.0 |
| | Final pH | 6.8±0.2 |
| **8.** | **Blood agar** | |
| | **Ingredients** | **g/L** |
| | Agar | 15.0 |
| | Beef extract | 10.0 |
| | Peptone | 10.5 |
| | Sodium chloride | 5.0 |
| | Final pH | 7.3±0.2 |
| **9.** | **MR VP medium (Himedium, India)** | |
| | **Composition** | 7.0 |
| | Buffered peptone | |
| | Dextrose | 5.0 |
| | Dipotassium phosphate | 5.0 |
| | Final pH(at 25°CO | 6.9±0.2 |
| **10.** | **Sugar media** | |
| | **a. Peptone water** | 10.0 gm |
| | Bacto-peptone | |
| | Sodium chloride | 5.0 gm |
| | 0.5% phenol red | 0. 1 ml |
| | Distilled water | 1000 ml |
| | **b. Sugar solutions** | 5 gm |
| | Individul sugar | 100 ml |
| | Distilled water | |
| | **c. Sugar mediua preparation** | 4.5 ml |
| | Pepton water | |
| | Sugar solution | 0.5 ml |
| **11.** | **Simmons citrare agar** | |
| | **Ingredients** | **g/L** |
| | Magnessium sulphate | 0.20 |
| | Ammunium dihydrogen phosphate | 1.0 |
| | Dipotassium phosphate | 1.0 |
| | Sodium citrate | 2.0 |

| | | |
|---|---|---|
| | Sodium chloride | 5.0 |
| | Bromothvmol blue | 0.08 |
| | Agar | 15.0 |
| **12.** | **TSI Agar slant** | |
| | **Ingredients** | 3.00 gm |
| | Lab Lamco Powder | |
| | Yeast extract | 3.00 gm |
| | Peptone | 20.00 gm |
| | Sodium chloride | 5.00 gm |
| | Lactose | 10.00 gm |
| | Sucrose | 10.00 gm |
| | Glucose | 1.00 gm |
| | Ferric citrate | 0.3 gm |
| | Sodium thiosulphate | 0.3 gm |
| | Phenol red | 0.3 gm |
| | Agar | 12.00 gm |
| | Distilled water | 1000 ml |

Preparation of reagents

| | | |
|---|---|---|
| **1**. | **Peptone water** | I gm |
| | peptone | |
| | Distilled water | 1000 ml |
| **2.** | **Kovacs reagent for indole preparation** | 5 gm |
| | P- dimethyl aminobenzal dehyde | |
| | Amyl alcohol | 75 gm |
| | Conc. HCL | 25 ml |

**3. V-P reagent-1**

5% alpha- naphthanol in absolute ethyl alcohol

**4**. **V-P reagent-2**

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath.When in solution, added 0.052 gm of cotton blue dye.

**5. Methyl red Solution**

| | |
|---|---|
| Methyl red | 0.05 gm |
| Ethanol(absolute) | 28 ml |
| Distilled water | 22 ml |

**6. Phenol red solution**

$0.2^0$o aqueous solution of phenol red

**7. Gram stain solutions**

a. **Stock crystal violet**

| | |
|---|---|
| crystal violet | 10 gm |
| Ethy1 alcohol | 1000m1 |

**b. Stock oxalate**

| | |
|---|---|
| Ammonium oxalate | 1 gm |
| Distilled water | 1000 ml |

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

**c. Lugol's Iodine solution**

| | |
|---|---|
| Iodine crystal | I gm |
| Potassium iodide | 2gm |

Dissolved completely in 10 ml of distilled water, then added to distilled water to make 300 ml. stored in ambar bottle.

| | | |
|---|---|---|
| **d.** | **Ethyl alcohol** | 250 ml |
| **e.** | **Acetone** | 250 ml |
| **f.** | **Counterstain** | 2.5 ml |
| | Safranine | |
| | Ethyl alcohol (95%) | 100 ml |

Safranine working solution:

The stock safranine is usually diluted as 1:4 with distilled water.