Characterisation of eco-friendly micro-organisms in the gut microflora of chicks

A Thesis submitted to the
Orissa University of Agriculture and Technology
in partial fulfilment of the requirements for the degree of
Master of Veterinary Science in
(Veterinary Microbiology)

By

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CERTIFICATE - I

This is to certify that the thesis entitled **"Characterisation of eco-friendly micro-organisms in the gut microflora of chicks"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Science (Veterinary Microbiology)** to the Orissa University of Agriculture and Technology is faithful record of *bona fide* and original research work carried out by **Pritam Pritiman Panda** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received by him from various sources during the course of investigation has been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Characterisation of eco-friendly micro-organisms in the gut microflora of chicks"** submitted by **Pritam Pritiman Panda** to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Veterinary Science (Veterinary Microbiology)** has been approved/ disapproved by the students' advisory committee and the external examiner.

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ABSTRACT

Eco-friendly bacterial communities within the gastrointestinal (GI) tract of chicks are a key component of gut health, resulting in optimal performance and the prevention of disease. So the present study was designed to characterize the commercial chicks baseline bacterial microbiome over time and across different regions of gastrointestinal tract such as jejunum, ileum, caecum, and colon. In this study 425 number of samples consisting of intestinal and faecal samples were collected from different established and non established poultry farms present in and around Bhubaneswar, Odisha which includes OUAT Instructional Livestock farm and Central Poultry Developmental Organisation (CPDO) during the period from September 2016 to May 2018 .The above samples were collected from day old chicks upto 4 weeks in weekly interval from four different breeds like Kalinga Brown, Banaraja, Colour Cross and Hubbard. The intestinal samples were examined across multiple ages (day 0,1st week, 2nd week, 3rd week and 4th week) for assessment of bacterial microbiome diversity using culture based assay. Similarly examination of faecal samples of different breeds was done for estimation of average bacterial load and maximum bacterial load was found in Hubbard breed at the age of 2nd and 4th week. General trends in the succession of the bacterial microbiome was observed such as decreasing populations of *Lactobacillus* and persistent prevalence of *Clostridium* in GI tract over ages. The study suggested that probiotics can be the most effective alternative for in feed antibiotics (IFAs) which can improve health and performance of chicks.

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ABBREVIATIONS USED

MLA	:	McConkey Lactose Agar
MHA	:	Mueller Hinton Agar
BHIB	:	Brain Heart Infusion Broth
CRA	:	Congo red agar
TSA	:	Tryptone soya agar
ml	:	milliliter
CCFA	:	Cycloserine Cefoxitin Fructose Agar
CFU	:	Colony Forming Unit
µ	:	Micrometer
hr(s)	:	hour(s)
° C	:	Degree Centigrade
BSM	:	Bifidus Selective Medium
g/L	:	Gram/Liter
Lit	:	Liter
spp.	:	species
et al.	:	Co-workers
BEA	:	Bile Esculin Azide
cm	:	centimeter
NCCLS	:	National Committee On Clinical Laboratory Standards
NaCl	:	Sodium chloride
Conc.	:	Concentration
pH	:	Hydrogen ion concentration

INTRODUCTION

The gastrointestinal tract (GIT) of humans and animals is believed to lack microbial colonization prior to birth (Isaacson and Kim, 2012), and thereafter experiences constant intimate contact with various microorganisms. This continuous microbial exposure and succession has resulted in evolutionary adaptations that manifest as distinct anatomical structures and physiological needs arising at certain ages. Through time, changes in the composition of the intestinal microbiome are believed to influence the overall health and performance of humans and animals. Initial reports describing GIT bacterial diversity were limited to those based on culture-dependent analyses using selective media that allow growth of cultivable microbes from the GIT. Using this method, the size of microbial populations was estimated to be 10^5 per gram of faeces (Allison et al., 1979; Russell, 1979) with Gram-positive bacteria, including *Lactobacillus* and *Clostridium*, which were found to be predominant (Robinson et al., 1981). However, a high percentage of all GIT bacterial species are not cultivable in vitro. Recently, in-depth investigation of GIT bacterial diversity has been facilitated with the advent of high-throughput pyrosequencing of the prokaryotic 16S rRNA libraries because it can provide in-depth and robust information on the microbiomes of various environments (Chang et al., 2011; Dowd et al., 2008).

The domestic chicken is a common model organism for human biological research and of course also forms the basis of a global protein industry. Recent methodological advances have spurred the recognition of microbiomes as complex communities with important influences on the health and disease status of the host. An overview of the current state of knowledge of the chicken gastrointestinal microbiome was provided focusing on spatial and temporal variability, the presence and importance of human pathogens, the influence of the microbiota on the immune system, and the importance of the microbiome for poultry nutrition. Review and meta-analysis of public data showed caecal communities dominated by Firmicutes and Bacteroides at the phylum level, while at finer levels of taxonomic resolution, a phylogenetically diverse assemblage of microorganisms appears to have similar metabolic functions that provide important benefits to the host as inferred from metagenomic data. This observation of functional redundancy may

have important implications for management of the microbiome. Advances are foreseen in strategies to improve gut health in commercial operations through management of the intestinal microbiota as an alternative to in-feed subtherapeutic antibiotics, improvements in pre- and probiotics, improved management of polymicrobial poultry diseases, and better control of human pathogens via colonization reduction or competitive exclusion strategies.

The importance of the composition of the microbiome has long been appreciated in poultry species and there is evidence that certain microbial populations may be positively associated with feed Apparent Metabolisable Energy (AME) in broiler chickens . However, there is a poor characterization of the microbiome of most commercial poultry species and the relationship of the microbiome to health and performance. Therefore, more research efforts are needed for functional characterization of the microbiome of poultry, observable performance and health status of the birds (Ajuwon et al.,2016).

Recently, the dual roles of microbiota in the gastrointestinal tract (GIT) have attracted attention: they support digestion of food, detoxification of certain compounds, growth performance and also provide nutrients. In addition, the GIT microbiota may play crucial roles in the health and immune system of chickens. In poultry species, the gastrointestinal tract microbiota is known to have some of the highest cell densities of any ecosystem with a range of 10^7 to 10^{11} bacteria per g of gut content . A diverse microbiota is found throughout the tract and is most extensive in the caecum. This creates one of the most complex ecosystems in nature. The composition of the microbiome determines consequence for host health and performance. In poultry, the absence of normal microflora in the caecum has been considered a major factor in the susceptibility of chicks to bacterial infection. Although the alimentary tract of the newly hatched chick is usually sterile, organisms rapidly gain access from the mother and the surrounding environment. Large numbers of anaerobic bacteria are capable of decomposing uric acid comprise the caecal flora of chicks 3 to 6 hours after hatching. During the first 2 to 4 days post hatch, *Streptococci* and *Enterobacteria* get colonized in the small intestine and caecum. After the first week, *Lactobacillus* predominate in the small intestine, and the caecum is colonized mainly by anaerobes (*Escherichia coli* and *Bacteroides*) with lower

numbers of facultative aerobes. A typical microflora of adult birds in the small intestine is established within 2 weeks, however, it was found that the adult caecal flora, which was mainly obligate anaerobes, took up to 30 days to develop. At that age, *Bifidobacteria* and *Bacteroides* predominate.

The gut represents a complex microbial ecosystem consisting of trillions of commensal bacteria living in symbiosis with the host. For chickens, interactions between the host and the gastrointestinal microbiome play a crucial role in host physiological development, health, nutrition, and food safety. As both 'the model and the system', the chicken microbiome offers important opportunities for both basic and applied research. As new tools continue to be applied to the chicken gastrointestinal microbiome, important progress will likely be made in several areas of poultry management.

Due to increase in the multidrug resistance microbes, antibiotic feed additives are being avoided around the globe as reported by several researchers. Knowing the microbial load in the various parts of the chicken intestine during various weeks of growth ,microbial modulation can be done in order to maintain proper growth so that there may not be any use of antibiotics as growth promoters.

Emphasis is being given to determine the complexity of chicken gut microbiota from day 0 to day 28 (4 weeks) which would reveal immune response of chickens during later stage of infection. Induction of eco-friendly bacteria to day old chicks at subsequent days caused modulation of the immune response by stimulating the innate immunity system which may prevent further colonisation of pathogenic microbes.

In order to achieve the above goal the experiment was conducted for isolation of different microorganisms (non pathogenic beneficial organisms) in chicken intestine from day 0 to 4 weeks. Microbial load of the beneficial organisms was estimated in chicks and further the gut microflora has been screened for biofilm production to develop a suitable age related microbial formulation.

REVIEW OF LITERATURE

Brian et al.,(2014) reviewed the chicken intestinal microbiome and further stated that The domestic chicken is a common model organism for human biological research and of course also forms the basis of a global protein industry. Recent methodological advances have spurred the recognition of microbiomes as complex communities with important influences on the health and disease status of the host. Review and meta-analysis of public data showed cecal communities dominated by Firmicutes and Bacteroides at the phylum level, while at finer levels of taxonomic resolution, a phylogenetically diverse assemblage of microorganisms appears to have similar metabolic functions that provide important benefits to the host as inferred from metagenomic data. Advances in strategies are foreseen to improve gut health in commercial operations through management of the intestinal microbiota as an alternative to in-feed subtherapeutic antibiotics, improvements in pre- and probiotics, improved management of polymicrobial poultry diseases, and better control of human pathogens via colonization reduction or competitive exclusion strategies.

Lu et al.,(2003) studied about the diversity and succession of the intestinal bacterial community of the maturing broiler chicken and further reported that the diversity of bacterial floras in the ilea and ceca of chickens that were fed a vegetarian corn-soy broiler diet devoid of feed additives was examined by analysis of 1,230 partial 16S rRNA gene sequences. Nearly 70% of sequences from the ileum were related to those of Lactobacillus, with the majority of the rest being related to Clostridiaceae (11%), Streptococcus (6.5%), and Enterococcus (6.5%). In contrast, Clostridiaceae-related sequences (65%) were the most abundant group detected in the cecum, with the other most abundant sequences being related to Fusobacterium (14%), Lactobacillus (8%), and Bacteroides (5%). Statistical analysis comparing the compositions of the different 16S rRNA libraries revealed that population succession occurred during some sampling periods. The significant differences among cecal libraries at 3 and 7 days of age, at 14 to 28 days of age, and at 49 days of age indicated that successions occurred from a transient community to one of increasing complexity as the birds aged. Similarly, the ileum had a stable bacterial community structure for birds at 7 to 21 days of age and between 21 to 28 days of age, but there was a very unique community structure at 3 and 49 days of age. It was also revealed that the composition of the ileal and cecal libraries did not significantly

differ when the birds were 3 days old, and in fact during the first 14 days of age, the cecal microflora was a subset of the ileal microflora. After this time, the ileum and cecum had significantly different library compositions, suggesting that each region developed its own unique bacterial community as the bird matured.

Yeoman et al.,(2012) studied about the microbiome of the chicken gastrointestinal tract and further stated that molecular techniques have led to significant gains in understanding of the chicken gastrointestinal microbiome. New advances, primarily in DNA sequencing technologies, have equipped researchers with the ability to explore these communities at an unprecedented level. A reinvigorated movement in systems biology offers a renewed promise in obtaining a more complete understanding of chicken gastrointestinal microbiome dynamics and their contributions to increasing productivity, food value, security, and safety as well as reducing the public health impact of raising production animals.

Oakley et al., (2013) studied about the Poultry-Associate Microbiome: Network Analysis and Farm-to-Fork Characterizations and further reported that microbial communities associated with agricultural animals are important for animal health, food safety, and public health. Analysis of longitudinal data following two flocks from the farm through processing showed a core microbiome containing multiple sequence types most closely related to genera known to be pathogenic for animals and/or humans, including Campylobacter, Clostridium, and Shigella. After the final stage of commercial poultry processing, taxonomic richness was ca. 2–4 times lower than the richness of fecal samples from the same flocks and Campylobacter abundance was significantly reduced. Interestingly, however, carcasses sampled at 48 hr after processing harboured the greatest proportion of unique taxa (those not encountered in other samples), significantly more than expected by chance. Among these were anaerobes such as Prevotella, Veillonella, Leptrotrichia, and multiple Campylobacter sequence types. Retail products were dominated by Pseudomonas, but also contained 27 other genera, most of which were potentially metabolically active and encountered in on-farm samples. Network analysis was focused on the foodborne pathogen Campylobacter and revealed a majority of sequence types with no significant interactions with other taxa, perhaps explaining the limited efficacy of previous attempts at competitive exclusion of Campylobacter.

Rehman et al.,(2007) studied about indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens further reported that the gastrointestinal The intestinal bacterial composition changes with age. The bacterial density of the small intestine increases with age and comprises of lactobacilli, streptococci, enterobacteria, fusobacteria and eubacteria. Strict anaerobes (anaerobic grampositive cocci, Eubacterium spp., Clostridium spp., Lactobacillus spp., Fusobacterium spp. and Bacteroides) are predominating caecal bacteria in young broilers. Data from culture-based studies showed that bifidobacteria could not be isolated from young birds, but were recovered from four-week-old broilers. Caecal lactobacilli accounted for 1.5 – 24% of the caecal bacteria. Gene sequencing of caecal DNA extracts showed that the majority of bacteria belonged to Clostridiaceae. Intestinal bacterial community is influenced by the dietary ingredients, nutrient levels and physical structure of feed. SCFA and other metabolic products are affected by diet formulation and age.tract is a dynamic ecosystem containing a complex microbial community. Feed composition and processing have great potential to influence the activities of intestinal bacteria towards a desired direction in order to support animal health, well-being and microbial safety of broiler meat.

Van der Wielen et al.,(2002) studied about spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth and further addressed the objective by using a 16S rDNA approach. PCR-amplicons from the V6 to V8 regions of the 16S rDNA of intestinal samples were separated by denaturing gradient gel electrophoresis (DGGE). The number of bands in all intestinal compartments increased when broilers grew older, indicating that the dominant bacterial community becomes more complex when chickens age. Each chicken had a unique banding pattern for all locations in the intestinal tract, irrespective of the age of chickens. This suggests that host-related factors affect the establishment of the dominant bacterial community. Banding patterns of intestinal compartments within one chicken were different from each other for broilers older than 4 days, except for both ceca which were highly similar. In 4-day-old broilers, banding patterns from crop, duodenum, and ileum were very similar. So he concluded that (unknown) host specific factors play an important role in the development of the intestinal bacterial community in each broiler chicken. Furthermore, compartment-specific factors play an important role in the bacterial development of each intestinal compartment within one chicken.

Bjerrum et al., (2006) studied about microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques and further reported that the microbial communities of the ileum and cecum of broiler chickens from a conventional and an organic farm were investigated using conventional culture techniques as well as cloning and sequencing of 16S rRNA genes. Eighty-five percent of the 557 cloned sequences were <97% related to known cultured species.The chicken ileum was dominated by lactobacilli, whereas the cecum harbored a more diverse microbial community.The cecum was dominated by a large group of bacteria with hitherto no close cultured relatives but most closely related to Faecalibacterium prausnitzii. Approximately 49 and 20% of the cecal clones belonged to this cluster inconventional and organic broiler chickens, respectively. The investigation was a descriptive rather than a comparative study of 2 different rearing systems; however, several differences were observed. For instance, *Clostridium perfringens* was found in significantly higher numbers in the birds from the organic farm compared with the conventional broilers, probably due to the addition of salinomycin to the conventional feed. In the ileum, the abundance of the different *Lactobacillus* species differed between the 2 broiler types. The culture-based and culture-independent techniques complemented each other well.

Zhong et al.,(2002) studied about 16S rRNA-based analysis of microbiota from the cecum of broiler chickens and further stated that the microbiota of the intestinal tract of chickens plays an important role in inhibiting the establishment of intestinal pathogens. Earlier culturing and microscopic examinations indicated that only a fraction of the bacteria in the cecum of chickens could be grown in the laboratory. Therefore, a survey of cecal bacteria was done by retrieval of 16S rRNA gene sequences from DNA isolated from the cecal content and the cecal mucosa. The ribosomal gene sequences were amplified with universal primers and cloned or subjected to temporal temperature gradient gel electrophoresis (TTGE). Partial 16S rRNA gene sequences were determined from the clones and from the major bands in TTGE gels. A total of 1,656 partial 16S rRNA gene sequences were obtained and compared to sequences in the GenBank. The comparison indicated that 243 different sequences were present in the samples. Overall, sequences representing 50 phylogenetic groups or subgroups of bacteria were found, but approximately 89% of the sequences represented just four phylogenetic groups (*Clostridium leptum*, *Sporomusa* sp., *Clostridium coccoides*, and enterics). Sequences of

members of the *Bacteroides* group, the *Bifidobacterium infantis* subgroup, and of *Pseudomonas* sp. each accounted for less than 2% of the total. Sequences related to those from the *Escherichia* sp. subgroup and from *Lactobacillus*, *Pseudomonas*, and *Bifidobacterium* spp. were generally between 98 and 100% identical to sequences already deposited in the GenBank. Sequences most closely related to those of the other bacteria were generally 97% or less identical to those in the databases and therefore might be from currently unknown species. TTGE and random cloning indicated that certain phylogenetic subgroups were common to all birds analyzed, but sequence data from random cloning also provided evidence for qualitative and quantitative differences among the cecal microbiota of individual birds reared under very similar conditions.

Guan et al.,(2003) studied about detection and identification of Lactobacillus species in crops of broilers of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis and further stated that the microflora of the crop was investigated throughout the broiler production period (0 to 42 days) using PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE) and selective bacteriological culture of lactobacilli followed by amplified ribosomal DNA restriction analysis (ARDRA). The birds were raised under conditions similar to those used in commercial broiler production. Lactobacilli predominated and attained populations of 10(8) to 10(9) CFU per gram of crop contents. Many of the lactobacilli present in the crop (61.9% of isolates) belonged to species of the Lactobacillus acidophilus group and could not be differentiated by PCR-DGGE. A rapid and simple ARDRA method was developed to distinguish between the members of the L. acidophilus group. HaeIII-ARDRA was used for preliminary identification of isolates in the L. acidophilus group and to identify Lactobacillus reuteri and Lactobacillus salivarius. MseI-ARDRA generated unique patterns for all species of the L. acidophilus group, identifying Lactobacillus crispatus, Lactobacillus johnsonii, and Lactobacillus gallinarum among crop isolates.

Pedroso et al.,(2005) studied about the structure of bacterial community in the intestines of newly hatched chicks and conducted a study to determine the structure of bacterial community in the small intestines of newly hatched chicks from 5 different populations at the moment they were delivered to the chicken house. The molecular technique, based on denaturing gradient gel electrophoresis of amplicons from 16S rDNA, more precise and efficient than traditional methods, was used. The total number of distinct amplicons, representing bacterial genotypes, found in the 5 populations was 48. Upon their arrival at

the farm, it was observed that 1-d-old broiler chicks carry a complex community of bacteria in their intestinal tract. The bacterial community may have been introduced in the intestinal tract of chicks in the prehatching phase, from the environment at the hatchery, or in transport.

Kabir et al.,(2009) studied about the role of probiotics in the poultry industry and further stated that the increase of productivity in the poultry industry has been accompanied by various impacts, including emergence of a large variety of pathogens and bacterial resistance. These impacts are in part due to the indiscriminate use of chemotherapeutic agents as a result of management practices in rearing cycles.He emphasises on the use of probiotics for prevention of bacterial diseases in poultry, as well as demonstrating the potential role of probiotics in the growth performance and immune response of poultry, safety and wholesomeness of dressed poultry meat evidencing consumer's protection, with a critical evaluation of results.

Wang et al.,(2017) studied about effect of probiotics on the meat favour and gut microbiota of chicken and tried to demonstrate the use of benefcial microbes for contributing to the favour characteristics and gut microbiota diversity of chicken.He selected s ix probiotics obtained from our laboratory and supplemented them in six different combinations to 420 newborn male Qingjiaoma chickens under the same controlled living environment (60 birds, no probiotic supplements). The results showed that chicken supplemented with Bacillus species showed beneficial effects in body weight. Acetate is the major fermentation production in the chicken caecum, and chicken supplemented with Pediococcus pentosaceus had the average higher short chain fatty acids (SCFAs) contents. In chicken caecal microfora, the abundance of Bacteroidetes bacteria was positively correlated with the content of propionate, butyrate, and isobutyrate, whereas an increase in acetate content was positively correlated to the abundance of Firmicutes. Compared to chickens without probiotic supplement, chickens supplemented with P. pentosaceus had more characteristic favour compounds in the sampled breast meat, especially higher concentrations of (E)-2-heptenal, (E,E)-2,4-nonadienal, and certain C6-C9 unsaturated fatty acids. This resulted in a stronger chicken-fatty or fatty odour which directly improved the favour. These findings suggest that probiotics can improve chicken meat favour and increase gut microbiota diversity.

Manafi et al.,(2018) studied about probiotic Bacillus species and Saccharomyces boulardii improve performance, gut histology and immunity in broiler chickens and further aimed to compare the effects of a new multispecies probiotic containing four Bacillus species and Saccharomyces boulardii (Microguard®) with a commercial probiotic (Protexin®) and a commonly used antibiotic in broilers. Six hundred one-day-old male Ross 308 broilers were randomized to six experimental treatments, with five replicates of 20 chicks each, for 42 days, receiving an ad libitum corn-soybean basal diet. Treatments were added to the basal diet and consisted of tetracycline as an antibiotic growth promoter (500 g/ton), three dosages of Microguard (50, 100 and150 g/ton) or Protexin (100 g/ton). The control group received the basal diet with no additive. The group fed with Microguard at 150 g/ton showed increased final bodyweight, weight gain, high density lipoprotein, triglyceride, and antibody titres against Newcastle disease (ND) and avian influenza (AI) levels. Improved feed conversion ratio, increased villus height, and villus highest crypt depth ratio, along with lower plasma gamma-glutamyl transpeptidase, alkaline phosphatase, alanine aminotransferase, were found in probiotic-supplemented broilers. Carcass yield, liver weights, breast muscle values, and abdominal fat weights were reduced in groups fed with 100 or 150 g/ton of Microguard. Caecal coliforms, Salmonella and Escherichia coli numbers decreased in groups fed with 100 or 150 g/ton of Microguard. These results show that Microguard at 150 g/ton is a promising probiotic to replace antibiotics in broiler feed as a growth-promoter while enhancing immune system responses and inducing beneficial modulations in the caecal microflora.

Dankowiakowska et al.,(2013) studied about probiotics, prebiotics and snybiotics in poultry – mode of action, limitation, and achievements and further stated that the withdrawal of antibiotic growth promoters from poultry industry have forced farmers to seek alternatives for the posing a risk factors of cross-resistance acquisition by harmful bacteria. A particular nuisance became salmonellosis and campylobacteriosis forcing to the elimination of whole poultry flocks as well as causing dangerous zoonotic diseases in humans. An excellent replacement for antibiotics have become the pro-, pre-and synbiotic substances which have a beneficial effect on the host organism through the development intensification of healthy intestinal microbial strains and the elimination of pathogenic strains. Such preparations may be administered both in the water spray as well as in feed. Excellent and promising method appears to be their injection directly into the egg air chamber in the 12th day of incubation. However, further studies are required to determine

the appropriate doses as well as combinations of bioactive substances and to determine the optimal way for their delivery.

Yang et al.,(2009) studied about dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics further stated that antibiotics have been added to poultry diets to maintain health and production efficiency in the last few decades. However, because of the development of resistance by pathogenic bacteria, which can impact on public health, antibiotics are being taken out of poultry diets around the world. The search for alternatives to replace IFAs has gained increasing interest in animal nutrition in recent years. Gut microflora has significant effects on host nutrition, health, and growth performance (Barrow, 1992) by interacting with nutrient utilization and the development of gut system of the host. This interaction is very complex and, depending on the composition and activity of the gut microflora, it can have either positive or negative effects on the health and growth of birds. Furthermore, he stated that gut microflora is a nutritional "burden" in fast-growing broiler chickens since an active microflora component may have an increased energy requirement for maintenance and a reduced efficiency of nutrient utilization. The focus of alternative strategies has been to prevent proliferation of pathogenic bacteria and modulation of indigenous bacteria so that the health, immune status and performance are improved.

Trafalska et al.,(2004) studied about Probiotics-An alternative for antibiotics and further reported that the condition and function of gastrointestinal (GI) tract are essential to our well being. Probiotics are defined as the microbial food supplements, which beneficially affect the host by improving its intestinal microbial balance. Probiotics are the functional food ingredients. They are used therapeutically to improve lactose tolerance and to prevent diarrhoea (especially viral diarrhoea in infants, antibiotic-associated diarrhoea, Clostridium difficile-associated diarrhoea and traveler's diarrhoea). Clinical studies suggest that probiotics might be useful in stimulation of the immune system, prevention of allergic diseases, control of GI tract inflammatory diseases and cancer prevention. Probiotic microbial species act by changing the composition of the gut microbiota.

Tortuero et al.,(1973) studied about influence of the implantation of Lactobacillus acidophilus in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora and further conducted experiments with broilers and Leghorn chicks

to ascertain the influence of Lact. acidophilli implantation (during the first five days of life) on the weight gain, feed conversion index, fat digestibility, nitrogen retention, ceca and feces weight and the lactic and Enterococci flora in chickens up to 15 days of age. The results have shown that, in comparison to the presence and effects of antibiotics, the Lactobacilli implantation resulted in a similar effect, manifested by increasing weight gain and better feed efficiency in chicks grown in an “old” environment. Although not significant, the differences in fat digestibility and nitrogen retention were also most apparent when the environment was “old.”.The implantation of Lact. acidophilus resulted in lower weights of the ceca and feces and a remarkable change in the bacterial flora of the ceca and the small intestine. At nine days of age of the chick, a marked increase in colonies of Lactobacilli was accompanied by an almost total disappearance of the Enterococci organisms.

Mountzouris et al.,(2007) evaluated the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities .The probiotic contained 2 Lactobacillus strains, 1 Bifidobacterium strain, 1 Enterococcus strain, and 1 Pediococcus strain. Four hundred 1-d-old male Cobb broilers were allocated in 4 experimental treatments for 6 wk. The experimental treatments received a corn-soybean basal diet and were as follows: "control," with no other additions; "probiotic in feed and water," (PFW) with probiotic administered at 1 g/kg of feed for the whole period and in water on scheduled intervals during the first 4 wk; "probiotic in feed," (PF) with probiotic in feed as in PFW; and "antibiotic," (AB) with addition of avilamycin at 2.5 mg/kg of feed. Salinomycin Na was used as a coccidiostat. Each treatment had 5 replicates of 20 broilers. Treatment effects on parameters of broiler performance and cecal microbial ecology were determined. Broiler BW, feed intake, and feed conversion ratio were determined on a weekly and overall basis. Cecal microflora composition, concentration of volatile fatty acids, and activities of 5 bacterial glycolytic enzymes (alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, and beta-glucuronidase) were determined at the end of the experiment. Overall, treatment PFW displayed a growth-promoting effect that did not differ from AB. Overall, feed conversion ratio in treatment AB was significantly better ($P < \text{or} = 0.01$) than the control treatment, whereas treatments PFW and PF were intermediate and not different from AB. Concentrations of bacteria belonging to Bifidobacterium spp., Lactobacillus spp., and

gram-positive cocci were significantly (P < or = 0.05) higher in treatments PFW and PF compared with the control and AB treatments. Treatments PFW and PF had significantly higher specific activities of alpha-galactosidase and beta-galactosidase compared with the control and AB treatments. In conclusion, probiotic treatment PFW displayed a growth-promoting effect that was comparable to avilamycin treatment. In addition, treatments PFW and PF modulated the composition and, to an extent, the activities of the cecal microflora, resulting imposition and metabolic activities by conducting an experiment.

Apata et al.,(2008) studied about growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of Lactobacillus bulgaricus and emphasised that probiotics are being developed for use in animal feed to enhance production performance and prevention of gastrointestinal infections. The ban on using antibiotics as growth promoters, antibiotic resistance and the inherent problems of developing new vaccines make a compelling case for developing alternatives for in-feed antibiotics. The alternatives of choice have to be considered under the environmental conditions of the animal. Among the probiotics in use today, Lactobacillus has been shown to play a vital role in disease prevention, immune enhancement, improved growth and carcass yield in poultry. The present study investigates the effect of Lactobacillus bulgaricus (LB)-based probiotic on the growth performance, nutrient digestibility and immune response of broilers under tropical environmental conditions. : Broilers fed LB diets consumed more feed (P < 0.05) and had greater body weight gain than the control group. Feed/gain ratio improved significantly (P < 0.05) with the 20, 40 and 60mg kg−1 LB diets compared with the control or 80 mg kg−1 LB diet. The apparent digestibilities of nitrogen and fat increased with LB supplementation. However, there was no significant difference (P > 0.05) in fibre digestibility. White blood cell count increased significantly in broilers fed higher levels (>40 mg kg−1) of LB compared with the control group. Antibody production measured as antibody titre against Newcastle disease vaccine showed a curvilinear response over the range of LB concentrations examined. The results indicate that LB addition to broiler chick diets significantly improved growth performance, increased nutrient digestibility and stimulated humoral immune response.

Netherwood et al.,(1999) studied about probiotics shown to change bacterial community structure in the avian gastrointestinal tract and further stated that culturing and molecular techniques were used to monitor changes in the bacterial flora of the avian gastrointestinal (GI) tract following introduction of genetically modified (GM) and

unmodified probiotics. Community hybridization of amplified 16S ribosomal DNA demonstrated that the bacterial flora of the GI tract changed significantly in response to the probiotic treatments. The changes were not detected by culturing. Although both GM and non-GM strains of Enterococcus faecium NCIMB 11508 changed the bacterial flora of the chicken GI tract, they did so differently. Probing the community DNA with an Enterococcus faecalis-specific probe showed that the relative amount of E. faecalis in the total eubacterial population increased in the presence of the non-GM strain and decreased in the presence of the GM probiotic compared with the results obtained with an untreated control group.

Owings et al.,(1990) studied the influence of dietary supplementation with Streptococcus faecium M-74 on broiler body weight, feed conversion, carcass characteristics, and intestinal microbial colonization and further conducted an experiment to determine the influence of Streptococcus faecium M-74 supplementation of broiler diets. Mixed-sex or male only chicks were used in the experiments. Both experiments consisted of six dietary treatments and four replicates per treatment in a randomized block design. The S. faecium was fed alone for 21, 36, or 44 days or for 44 days with S. faecium supplemented in the water for the first 14 days. In another treatment, S. faecium was fed for 44 days, but the feed was restricted for Days 8 through 13. The S. faecium was also fed in combination with antibacterial products (AP) for 44 days. An additional diet was an unsupplemented basal, and another was supplemented only with AP. In Experiment 1, feed efficiency was significantly better with the basal and diets supplemented with S. faecium than with those diets supplemented with AP or AP and S. faecium. In Experiment 2, BW of broilers at 44 days of age were significantly heavier for broilers receiving S. faecium in the feed and also S. faecium in the water for the first 14 days as compared with broilers receiving AP or AP and S. faecium supplementation. There were no significant differences in carcass yield or composition characteristics. In Experiment 1, the scores representing S. faecium colonies found in the intestinal tract were not influenced by dietary treatment. The ceca had the highest S. faecium score of any of the intestinal tract locations.

Ilabaca et al.,(2016) studied about biofilm forming Lactobacillus: new challenges for the development of probiotics and further stated that probiotics are live bacteria, generally administered in food, conferring beneficial effects to the host because they help to prevent or treat diseases, the majority of which are gastrointestinal. Numerous investigations have

verified the beneficial effect of probiotic strains in biofilm form, including increased resistance to temperature, gastric pH and mechanical forces to that of their planktonic counterparts. In addition, the development of new encapsulation technologies, which have exploited the properties of biofilms in the creation of double coated capsules, has given origin to fourth generation probiotics. Reviews had so far focused on the detrimental effects of biofilms associated with pathogenic bacteria. Therefore he aimed to amalgamate information describing the biofilms of Lactobacillus strains which are used as probiotics, particularly L. rhamnosus, L. plantarum, L. reuteri, and L. fermentum. Additionally, we have reviewed the development of probiotics using technology inspired by biofilms.

Jones et al.,(2009) studied about probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors and emphasised that commensal-derived probiotic bacteria inhibit enteric pathogens and regulate host immune responses in the gastrointestinal tract. Lactobacillus reuteri formed biofilms that retained functions potentially advantageous to the host including modulation of cytokine output and the production of the antimicrobial agent, reuterin. Immunomodulatory activities of biofilms were demonstrated by the abilities of specific L. reuteri strains to suppress human TNF production by LPS-activated monocytoid cells. Quantification of the antimicrobial glycerol derivative, reuterin, was assessed in order to document the antipathogenic potential of probiotic biofilms. L. reuteri biofilms differed in the quantities of reuterin secreted in this physiological state.He further concluded that L. reuteri biofilms secreted factors that confer specific health benefits such as immunomodulation and pathogen inhibition. Future probiotic selection strategies should consider a strain's ability to perform beneficial functions as a biofilm.

Aoudia et al.,(2014) studied about Biofilms of Lactobacillus plantarum and Lactobacillus fermentum: Effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties and extensively investigated probiotic functions associated with biofilms.He showed that strains of Lactobacillus plantarum and Lactobacillus fermentum are able to grow as biofilm on abiotic surfaces, but the biomass density differs between strains. Microtiter plate biofilm assays were performed under growth conditions mimicking to the gastrointestinal environment. Osmolarity and low concentrations of bile significantly enhanced Lactobacillus spatial organization. Two L. plantarum strains were able to form biofilms under high concentrations of bile and mucus.

We used the agar well-diffusion method to show that supernatants from all Lactobacillus except the NA4 isolate produced food pathogen inhibitory molecules in biofilm. Moreover, TNF-a production by LPS-activated human monocytoid cells was suppressed by supernatants from Lactobacillus cultivated as biofilms but not by planktonic culture supernatants. However, only L. fermentum NA4 showed anti-inflammatory effects in zebrafish embryos fed with probiotic bacteria, as assessed by cytokine transcript level (TNF-a, IL-1b and IL-10). We conclude that the biofilm mode of life is associated with beneficial probiotic properties of lactobacilli, in a strain dependent manner. Those results suggest that characterization of isolate phenotype in the biofilm state could be additional valuable information for the selection of probiotic strains.

Fernandez et al.,(2015) studied characterisation of biofilms formed by Lactobacillus plantarum WCFS1 and food spoilage isolates and further reported that Lactobacillus plantarum has been associated with food spoilage in a wide range of products and the biofilm growth mode has been implicated as a possible source of contamination.H e analysed that the biofilm forming capacity of L. plantarum WCFS1 and six food spoilage isolates. Biofilm formation as quantified by crystal violet staining and colony forming units was largely affected by the medium composition, growth temperature and maturation time and by strain specific features. All strains showed highest biofilm formation in Brain Heart Infusion medium supplemented with manganese and glucose. For L. plantarum biofilms the crystal violet (CV) assay, that is routinely used to quantify total biofilm formation, correlates poorly with the number of culturable cells in the biofilm. This can in part be explained by cell death and lysis resulting in CV stainable material, conceivably extracellular DNA (eDNA), contributing to the extracellular matrix. The strain to strain variation may in part be explained by differences in levels of eDNA, likely as result of differences in lysis behaviour. In line with this, biofilms of all strains tested, except for one spoilage isolate, were sensitive to DNase treatment. In addition, biofilms were highly sensitive to treatment with Proteinase K suggesting a role for proteins and/or proteinaceous material in surface colonisation. This study shows the impact of a range of environmental factors and enzyme treatments on biofilm formation capacity for selected L. plantarum isolates associated with food spoilage, and may provide clues for disinfection strategies in food industry.

Walencka et al.,(2008) studied the influence of Lactobacillus acidophilus-derived surfactants on staphylococcal adhesion and biofilm formation and further stated that the

ability of surfactants obtained from three Lactobacillus acidophilus strains to inhibit Staphylococcus aureus and S. epidermidis biofilms was evaluated. Their influence was determined on bacterial initial adhesion, biofilm formation and dispersal using MTT-reduction assay, confocal laser scanning microscopy and image PHLIP analysis. The number of adhering S. aureus and S. epidermidis cells after a 3-h co-incubation with biosurfactants was reduced by 5-56 % in a strain-and dose-dependent manner. S. epidermidis-and, to a lower extent, in S. aureus-biofilm formation was also inhibited in the presence of the tested surfactants. The addition of surfactants to preformed mature biofilms accelerated their dispersal, and changed the parameters of biofilm morphology. The L. acidophilus-derived surfactants inhibit bacterial deposition rate and biofilm development (and also its maturation) without affecting cell growth probably due to the influence on the cell-surface hydrophobicity of staphylococci.

Kubota et al.,(2008) studied about biofilm formation by lactic acid bacteria and resistance to environmental stress and further investigated the formation of biofilms by 3 type strains of lactic acid bacteria (LAB), Lactobacillus plantarum, Lactobacillus brevis and Lactobacillus fructivorans, as representatives of LAB that cause food deterioration or contamination. Lactobacillus plantarum subsp. plantarum JCM1149 and Lactobacillus brevis JCM1059 appeared to adhere and accumulate on glass cover slips. Lactobacillus fructivorans JCM1117 cells made thin cellophane-like biofilms, and most of the biofilm cells became longer than the planktonic cells. He tested the resistance of biofilm and planktonic L. plantarum subsp. plantarum JCM1149 cells to acetic acid and ethanol, which strongly inhibit the growth of bacteria and are important in food preservation. The biofilm cells were more resistant than the planktonic cells and the surfaces of the treated planktonic cells were badly damaged, whereas those of the biofilm cells were only slightly damaged.He isolated 43 LAB from onions and the biofolm cells of an isolate, L. plantarum M606 also had high resistance. These results demonstrate the significance of studying biofilms of LAB in the food industry.

Anderson et al.,(2010) studied that Lactobacillus plantarum DSM 2648 is a potential probiotic that enhances intestinal barrier function and further aimed to identify bacterial isolates having the potential to improve intestinal barrier function. Lactobacillus plantarum strains and human oral isolates were screened for their ability to enhance tight junction integrity as measured by the transepithelial electrical resistance (TEER) assay. Eight commercially used probiotics were compared to determine which had the greatest

positive effect on TEER, and the best-performing probiotic strain, Lactobacillus rhamnosus HN001, was used as a benchmark to evaluate the isolates. One isolate, L. plantarum DSM 2648, was selected for further study because it increased TEER 135% more than L. rhamnosus HN001. The ability of L. plantarum DSM 2648 to tolerate gastrointestinal conditions and adhere to intestinal cells was determined, and L. plantarum DSM 2648 performed better than L. rhamnosus HN001 in all the assays. Lactobacillus plantarum DSM 2648 was able to reduce the negative effect of Escherichia coli [enteropathogenic E. coli (EPEC)] O127:H6 (E2348/69) on TEER and adherence by as much as 98.75% and 80.18%, respectively, during simultaneous or prior coculture compared with EPEC incubation alone.SC

Gunal et al.,(2006) studied the effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers and further stated the effects of an antibiotic growth promoter (flavomycin), a probiotic mixture (protexin) or a mixture of organic acids including plant extract and mineral salts (genex) on performance, intestinal microbial flora and tissue morphology have been examined in 160 day-old Ross 308 broiler chicks. Commercial corn-soybeanbased broiler starter and grower diets were formulated as basal diets for control treatment. Basal diets were supplemented with a probiotic (0.1% protexin), an antibiotic growth promoter (0.1% flavomycin), an organic acids mixture (0.2% genex) or a combination of a probiotic with an organic acids mixture (0.1% protexin+0.2% genex). In total, five dietary treatments were employed in the trial. Live weight gain, feed intake, feed conversion ratio and mortality were not affected by dietary treatments throughout the experiment. However, relative weight of the small intestine of antibiotic treatment had significantly less than that of the basal diet. Intestinal microbial flora and tissue were determined at 21 and 42 days. In both periods, antibiotic or organic acids mixture treatments significantly decreased total bacteria counts. In addition to that all treatments significantly decreased gram negative bacteria counts compared to the basal diet. Probiotic treatment significantly increased ileum and jejunum villus height, whereas antibiotic treatment significantly decreased muscularis thickness compared to the basal diet.

Bai et al.,(2013) studied the effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens and further investigated the effects of a probiotic product incorporating Lactobacillus fermentum and Saccharomyces cerevisiae on the growth performance and intestinal immune status in

broiler chickens. A total of six hundred ninety-six 1-d-old male Cobb broilers were randomly allotted by BW in 1 of 4 treatments for 6-wk trial. The dietary treatments included the basal diet (NC), and the basal diets supplemented with an antibiotic (100 mg of chlortetracycline/kg of diet; PC), 0.1%, or 0.2% probiotic product (containing 1 × 10(7) cfu/g of Lactobacillus fermentum JS and 2 × 10(6) cfu/g of Saccharomyces cerevisiae). Each treatment had 6 replicates with 29 broilers each. The ADG and feed efficiency were improved ($P < 0.05$) in broilers fed the probiotic diet compared with NC, and were similar to the PC group during 1 to 21 d. However, there were no significant differences in growth performance of broilers during 22 to 42 d among different dietary treatments. Chicks fed probiotics had higher proportions of CD3+, CD4+, and CD8+ T-lymphocytes, whereas the antibiotic diet decreased the proportion of CD8+ T-lymphocytes in the foregut of broilers at 21 and 42 d compared with the NC group. No significant difference was observed in the mRNA expression level of chicken B-cell marker chB6 (Bu-1) in the foregut of chickens among different treatments. Probiotic-supplemented diets increased ($P < 0.05$) the mRNA expression levels of Toll-like receptor (TLR) 2 and TLR 4 at 21 d, and only the TLR2 mRNA level at 42 d in the foregut of chickens, but did not change ($P > 0.05$) TLR7 mRNA expression compared with NC or PC. There was no significant difference in the above TLR mRNA levels in the intestine of broilers between PC and NC. These results indicated that the probiotic product incorporating Lactobacillus fermentum and Saccharomyces cerevisiae could stimulate intestinal T-cell immune system without decreasing growth performance in broilers during 1 to 21 d.

Awad et al.,(2009) studied the effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens and conducted an experiment to investigate the effects of dietary supplementations of synbiotic and probiotic on broiler performance, carcass yield, organs weights, and histomorphological measurements of small intestine. Six hundred 1-d-old broiler chicks were randomly assigned to 1 of 3 dietary treatments for 5 wk. The dietary treatments were 1) control, 2) basal diets supplemented with synbiotic (1 kg of Biomin IMBO/ ton of the starter diets and 0.5 kg/ton of the grower diets), 3) basal diets supplemented with probiotic (1 kg of a homofermentative and a heterofermentative Lacto-bacillus sp./ton of feed). The BW, average daily weight gain, carcass yield percentage, and feed conversion rate were significantly ($P < 0.05$) increased by the dietary inclusion of the synbiotic

compared with the control and probiotic-fed broilers. Moreover, a slight improvement in performance traits was observed in broilers fed the probiotic compared with control birds. The absolute and relative weight of spleen and thymus tended to be greater ($P < 0.1$) for the probiotic-supplemented group compared with the synbiotic-supplemented group. The relative liver weight was greater ($P < 0.05$) for probiotic-fed birds compared with synbiotic-fed birds. Additionally, the weight of small intestine was greater for either probiotic- (3.17) or synbiotic-fed birds (3.11) than the controls (2.89). Furthermore, dietary treatments influenced the histomorphological measurements of small intestinal villi. The addition of either probiotic or synbiotic increased ($P < 0.05$) the villus height:crypt depth ratio and villus height in both duodenum and ileum. The duodenal crypt depth remained unaffected ($P > 0.05$). However, the ileal crypt depth was decreased by dietary supplementations compared with control. In conclusion, synbiotic or probiotic displayed a greater efficacy as growth promoters for broilers. Furthermore, the dietary supplementations resulted in an increase in the villus height and crypt depth of intestinal mucosa of broilers. The increase in the villus height and villus height:crypt depth ratio was associated with improvement of growth performance for both synbiotic and probiotic. This indicates that the synbiotic and probiotic can be used as a growth promoter in broiler diets and can improve the gut health. These products show promising effects as alternatives for antibiotics as pressure to eliminate growth-promotant antibiotic use increases.

Ashayerizadeh et al.,(2009) studied effect of dietary antibiotic, probiotic and prebiotic as growth promoters, on growth performance, carcass characteristics and hematological indices of broiler chickens and conducted an experiment to compare the effects of antibiotic (flavomycin), probiotic (primalac), prebiotic (Biolex-MB) and mixture of probiotic and prebiotic (primalac plus Biolex-MB) as dietary growth promoter on growth performance, carcasscharacteristics and hematological indices of broiler chickens. Three hundred day old Ross 308 broilers were equally distributed into 30 floor pens and reared for 42 day. A basal diet was formulated covering the recommendations of NRC (1994) for starter (0-21 days) and grower (22-42 days) periods and considered as control diet. Four tested diets were formulated by supplemented the basal control diet with antibiotic (flavomycin), probiotic (primalac), prebiotic (Biolex-MB) and mixture of primalac plus Biolex-MB, respectively. Six replicates were used for each treatment. The results of present study showed that all growth promoters used was improved growth indices of

Ross 308 broilers. The highest significant ($p<0.05$) values of carcass and thigh were recorded for broilers fed diet supplemented with flavomycin. The highest ($p>0.05$) value of breast was recorded for broilers fed the diet supplemented with primalac, meanwhile the lower value were showed for birds fed either diet or diet supplemented with Biolex-MB. The percent of carcass and cuts followed the same trend. Hematological parameter including cholesterol was recorded the highest ($p>0.05$) values groups fed the diets either control or supplemented with flavomycin, meanwhile the lower value was showed for bird fed diet supplemented primalac plus Biolex-MB. Triglycerides and very low density lipoprotein cholesterol (VLDL) were recorded the highest concentration for bird fed both control and diet supplemented with flavomycin groups while least concentration was found for bird fed diet supplemented with primalac. The results this study revealed that probiotic and prebiotic as growth promoters can use as alternatives non-antibiotic feed additives to their free harmful side effects on the consumers and to improve broiler chickens growth indices.

Patterson et al.,(2003) studied application of prebiotics and probiotics in poultry production and further stated that the intestinal microbiota, epithelium, and immune system provide resistance to enteric pathogens.He suggested that resistance is not solely due to the sum of the components, but that cross-talk between these components is also involved in modulating this resistance. Inhibition of pathogens by the intestinal microbiota has been called bacterial antagonism, bacterial interference, barrier effect, colonization resistance, and competitive exclusion. Mechanisms by which the indigenous intestinal bacteria inhibit pathogens include competition for colonization sites, competition for nutrients, production of toxic compounds, or stimulation of the immune system. These mechanisms are not mutually exclusive, and inhibition may comprise one, several, or all of these mechanisms. Consumption of fermented foods has been associated with improved health, and lactic acid bacteria (lactobacilli and bifidobacteria) have been implicated as the causative agents for this improved health. Lactic acid bacteria and certain other microorganisms can increase resistance to disease and that lactic acid bacteria can be enriched in the intestinal tract by feeding specific carbohydrates. Increased bacterial resistance to antibiotics in humans has caused an increase in public and governmental interest in eliminating sub-therapeutic use of antibiotics in livestock. An alternative approach to sub-therapeutic antibiotics in livestock is the use of probiotic microorganisms, prebiotic substrates that enrich certain bacterial populations, or synbiotic

combinations of prebiotics and probiotics.His research was focused on identifying beneficial bacterial strains and substrates along with the conditions under which they are effective.

Kratzer et al.,(1983) studied the effect of oral dosing of Lactobacillus strains on gut colonization and liver biotin in broiler chicks and further reported that, chicks dosed with Lactobacillus strains had lower numbers of coliforms in caecal macerates than the control strains of host specific (strain KTM, 74/1, and 59) and non host specific (strain 40) lactobacilli were orally dosed to day-old broiler chicks. Dose levels consisted of: 5.0, 7.0, and 9.0 log10 cfu/day for 21 days. Each treatment and control group (placebo, no culture) contained three replications of 6 chicks in Experiment 1 (strains 40 and KTM) and 7 chicks in Experiment 2 (strains 74/1 and 59). Dosing of 7.0 log10 cfu and above tended to depress chick growth. Faecal shedding of lactobacilli were the same (8.8 log10 cfu) for all dose levels on Day 5 but decreased on Day 14 for strains KTM and 40. For strains 74/1 and 59 at all dose levels, the faecal shedding of lactobacilli was 9.9 log10 cfu for Day 5 and 8.5 log10cfu for Day 14. Microbiology was performed on contents of the crop and ileum and on the tissue macerates from the crop, ileum, and ceca from chicks killed on Days 7 and 21 of the experiments. Chicks dosed with strains 59 and KTM had lower numbers of coliforms in caecal macerates than the control and chicks dosed with strains 40 and 74/1. No significant differences were observed in lactobacilli found in contents and tissues between 5.0 and 9.0 log10 cfu for all lactobacilli strains dosed. There was a trend for the liver biotin to be higher in chicks dosed with 7.0 log10 cfu than chicks dosed with 5.0 or 9.0 log10 cfu or no culture, but the difference was not significant (P>.05).

Watkins et al., (1982) studied in vivo effects of Lactobacillus acidophilus against pathogenic Escherichia coli in gnotobiotic chicks and observed that, competitive exclusion of pathogenic E. coli occurred in the gastrointestinal tract of gnotobiotic chicks dosed with L. acidophilus.Chicks were hatched germfree in gnotobiotic isolators to determine the inhibitory effects of Lactobacillus acidophilus towards pathogeneic Escherichia coli in vivo. Twelve trials were conducted in two flexible film isolators utilizing a total of 221 chicks. One treatment consisted of inoculating 2-day-old chicks with L. acidophilus, then challenging with pathogenic E. coli with subsequent dosing with L. acidophilus. The other treatment consisted of challenging with the E. coli at 2 days of age, then subsequently dosing with L. acidophilus. Statistical analysis of the data showed initial dosing with L. acidophilus prevented excessive mortality when chicks

were challenged with E. coli. Also, continued dosing with L. acidophilus lowered the pH in the crop, cecum, and rectum whether chicks were initially given L. acidophilus or E. coli. This strain of L. acidophilus was capable of competing with E. coli in the gut of gnotobiotic chicks.

Midilli et al., (2008) studied the effects of dietary probiotic and prebiotic supplementation on growth performance and serum IgG concentration of broilers and showed the ineffectiveness of additive supplementation of probiotics on systemic IgG.The study was conducted to investigate the effects of probiotic and/or prebiotic supplementation on growth performance and serum IgG concentrations in broilers. One thousand two hundred one-day old Ross-308 broiler chicks of mixed sex were randomly divided into four treatment groups of 300 birds each. The treatments were: Starter diets: 1) Unsupplemented control diet; 2) Probiotic (Bio-Plus 2B® 0.05%); 3) Prebiotic (Bio-Mos® 0.2%); 4) Probiotic and Prebiotic mixture (Bio-Plus 2B® 0.05% and Bio-Mos® 0.2%). The grower diets were: 1) Control with no supplements; 2) Probiotic (Bio-Plus 2B® 0.05%); 3) Prebiotic (Bio-Mos® 0.1%); 4) Probiotic and Prebiotic mixture (Bio-Plus 2B® 0.05% and Bio-Mos® 0.1%). Each treatment group was further sub-divided into five replicates of 60 birds per replicate. The chicks were fed the broiler starter diet for the first 21 d and the broiler grower diet between days 22 and 42. Dietary probiotic and/or prebiotic supplementation did not significantly affect body weight, body weight gain, feed intake, carcass weight, carcass yield or concentration of immunoglobulin (IgG) in the serum. However, feed conversion ratio was improved significantly in the supplemented treatments compared to the unsupplemented control. Probiotic and/or prebiotic supplementation did not significantly affect any of the examined parameters except for an improved feed conversion ratio.

Fuller (1977) studied the importance of lactobacilli in maintaining normal microbial balance in the crop andfound that host-specific Lactobacillus strains were able to decrease Escherichia coli in the crop and small intestine.The effect of lactobacilli on Escherichia coli has been examined in vitro and in chicken crop in vivo. Inhibition of E. coli was dependent on the presence of sufficient numbers of lactobacilli. In a standard test some lactobacilli were bacteriostatic and one was bactericidal. The bacteriostasis was due to the low pH produced by these strains but bactericidal activity could not be accounted for by pH alone. In gnotobiotic animals the bactericidal strain was no more inhibitory for E. coli than was a bacteriostatic strain.

Kizerwetter-Swida et al. (2009) studied the protective effect of potentially probiotic Lactobacillus strain on infection with pathogenic bacteria in chickens and reported that L. salivarius 3d strain reduced the number of Salmonella enteritidis and Clostridium perfringens in the group of chickens treated with Lactobacillus.The probiotic potential of a Lactobacillus salivarius 3d strain isolated from chicken faeces was assessed in one day old chickens. Lactobacillus salivarius 3d was administered per os at a concentration of 10(8) cfu in 100 microl of PBS. The chickens were then challenged with pathogenic bacteria: Salmonella Enteritidis, Campylobacter jejuni and Clostridium perfringens. Samples of caecal contents and livers were collected after 1, 2, 3, 7 and 14 days after infection. Lactobacilli and pathogenic bacterial cell counts were determined in the samples. The study showed that L. salivarius 3d reduced the number of Salmonella Enteritidis and Clostridium perfringens in the group of chickens treated with Lactobacillus. It was concluded that L. Salivarius 3d might be used as a potential probiotic for chickens.

Brisbin et al., (2008) studied the gene expression profiling of chicken lymphoid cells after treatment with Lactobacillus acidophilus cellular components and investigated spatial and temporal expression of immune system genes in chicken caecal tonsil and spleen mononuclear cells in response to structural constituents of L. acidophilus and they found that caecal tonsil cells responded more rapidly than spleen cells to the bacterial stimuli, with the most potent stimulus for caecal tonsil cells being DNA and for splenocytes being the bacterial cell wall components. It was also discovered that in both splenocytes and cecal tonsil cells, STAT2 and STAT4 genes were highly induced and the expression of STAT2, STAT4, IL-18, MyD88, IFN-alpha, and IFN-gamma genes were up-regulated in caecal tonsil cells after treatment with L. acidophilus DNA.

Ehrmann et al., (2002) studied about characterization of lactobacilli towards their use as probiotic adjuncts in poultry and further reported that a total of 112 strains of lactic acid bacteria of duck origin were studied for their use as a probiotic feed supplement. In vitro studies included aggregation, co-aggregation, cell surface hydrophobicity and adhesion activities on poultry crop cells and human Hep2-cells. Additionally, growth with bile acids (chicken bile, ox gall and taurocholic acid) and tolerance to acidic pH were tested. Among all the isolates, two strains (Lactobacillus animalis TMW 1.972 and Lactobacillus

salivarius TMW 1.992) were selected for a survival test in poultry. Monitoring and differentiation of these strains was achieved by selective detection as rifampicin and erythromycin double-resistant mutants. After a single feed administration, both micro-organisms were shown to persist in the crop and caecum of ducks for a period of 18 and 22 days, respectively. For identification of Lact.animalis and Lact. salivarius, two specific PCRs targeted against 16S rDNA were developed. Within the autochtoneous microflora of ducks, two strains of lactobacilli exhibited strong potential as probiotic adjuncts. The results indicated that the natural gut microflora of poultry serves as an excellent source for optimal strains. A general strategy for the selection of probiotic strains was presented. The suggested sequence of tests allowed identification of the most promising candidates within complex ecosystems or large strain collections with minimal expenditure.

Koenen et al.,(2004) studied the development and validation of a new in vitro assay for selection of probiotic bacteria that express immune-stimulating properties in chickens in vivo and further reported thatoral administration of immunoprobiotic bacteria may support animal health. Species specificity of such microorganisms requires appropriate selection. An in vitro assay for the selection of immunoprobiotic lactic acid bacteria was developed in chicken. The assay allowed testing of large numbers of individual strains. Immune stimulation in vitro correlated well with the in vivo situation in two experiments and no false negative results occurred. The assay was an appropriate selection tool for immunomodulating properties of lactic acid bacteria in chicken.

S F Barefoot et al.,(1983) studied the detection and activity of lactacin B, a bacteriocin produced by Lactobacillus acidophilusand further reported thata total of 52 strains of Lactobacillus acidophilus were examined for production of bacteriocins. A majority (63%) demonstrated inhibitory activity against all members of a four-species grouping of Lactobacillus leichmannii, Lactobacillus bulgaricus, Lactobacillus helveticus, and Lactobacillus lactis. Four L. acidophilus strains with this activity also inhibited Streptococcus faecalis and Lactobacillus fermentum, suggesting a second system of antagonism. Under conditions eliminating the effects of organic acids and hydrogen peroxide, no inhibition of other gram-positive or -negative genera was demonstrated by L. acidophilus. The agent produced by L. acidophilus N2 and responsible for inhibition of L.

leichmannii, L. bulgaricus, L. helveticus, and L. lactiswas investigated. Ultrafiltration studies indicated a molecular weight of approximately 100,000 for the crude inhibitor. The agent was sensitive to proteolytic enzymes and retained full activity after 60 min at 100 degrees C (pH 5). Activity against sensitive cells was bactericidal but not bacteriolytic. These characteristics identified the inhibitory agent as a bacteriocin, designated lactacin B.

Chateau et al.,(1993) studied about distribution of pathogen inhibition in the Lactobacillus isolates of a commercial probiotic consortium and further reported that pure strains of Lactobacillus ssp. isolated from a commercial probiotic consortium were checked in a double layer solid medium for their inhibition activities against selected pathogenic bacteria including serotypes of Listeria monocytogenes, Escherichia coli and Salmonella. The antagonistic properties of the Lactobacillus strains might be related to the production of bacteriocin-like compounds. All the pathogens tested were inhibited by one or a few strains of Lactobacillus, the best inhibition was observed against L. monocytogenes but the inhibition was also satisfactory against E. coli, Salm. typhimurium and Salm. enteritidis.

Monica Pascual et al.,(1999) reported that Lactobacillus salivarius CTC2197 prevents salmonella enteritidis colonization in chickens. A rifampin-resistant Lactobacillus salivarius strain, CTC2197, was assessed as a probiotic in poultry, by studying its ability to prevent Salmonella enteritidis C-114 colonization in chickens. When the probiotic strain was dosed by oral gavage together with S. enteritidis C-114 directly into the proventriculus in 1-day-old Leghorn chickens, the pathogen was completely removed from the birds after 21 days. The same results were obtained when the probiotic strain was also administered through the feed and the drinking water apart from direct inoculation into the proventriculus. The inclusion of L. salivarius CTC2197 in the first day chicken feed revealed that a concentration of 105 CFU g−1 was enough to ensure the colonization of the gastrointestinal tract of the birds after 1 week. However, between 21 and 28 days, L. Salivarius CTC2197 was undetectable in the gastrointestinal tract of some birds, showing that more than one dose would be necessary to ensure its presence till the end of the rearing time. Freeze-drying and freezing with glycerol or skim milk as cryoprotective agents, appeared to be suitable methods to preserve the probiotic strain. The inclusion of the L. salivarius CTC2197 in a commercial feed mixture seemed to be a good way to supply it on the farm, although the strain showed sensitivity to the

temperatures used during the feed mixture storage and in the chicken incubator rooms. Moreover, survival had been improved after several reinoculations in chicken feed mixture.

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MATERIALS AND METHODS

In the present study four hundred twenty five samples (n=425) consisting of intestinal and faecal samples were collected from different established and non - established poultry farms present in and around Bhubaneswar, Odisha which includes OUAT Instructional Livestock farm and Central Poultry Developmental Organisation(CPDO) during the period from September 2016 to May 2018 .The above samples were collected from day old chicks upto 4 weeks in weekly interval from different breeds like Kalinga Brown, Banaraja, Colour Cross and Hubbard.

After humane method of slaughter of the birds for the greater cause of science different parts of the intestine were aseptically collected and subsequently processed for routine isolation and identification of different microorganisms.

3.1 Sample collection:

In the present study 85 number of birds of four different breeds such as Kalinga Brown, Colour Cross, Banaraja and Hubbard were selected and humanely slaughtered at five time points i.e. day old,one week, two weeks, three weeks and four weeks of age. Samples (n=425) were collected and pooled for each flock of various breeds. Various sections of the small and large intestine i.e. jejunum, ileum,caecum and colon were aseptically collected and homogenised. The homogenates included intestinal contents which were stored at 4^0C for further assessment of total bacterial content.

3.2 Media used:

Various synthetic media were employed in the current study. The various media included i.e. Nutrient broth, Brain heart infusion (BHI) broth, McConkey lactose agar (MLA), EMB agar (Eosin methylene blue agar), Mueller Hinton Agar (MHA), Tryptone soya agar (TSA), MRS agar, BSM agar, BEA agar, Hoyle's tellurite agar, CCFA agar, Wilkins Chalgren agar, Luria-Bertani Broth w/o Nacl, Thioglycolate broth Congo red agar (CRA), Brilliant green bile broth and BSM broth were obtained from HiMedia, Mumbai . The media were prepared according to the manufacturer's instructions.

3.3 Estimation of bacterial load of intestinal and faecal content:

One gram of intestine content was first put with a sterile stick in a sterile measuring tube and inoculated in 5 ml. of BHI broth. After mixing thoroughly they were incubated for 24 hrs at 37^0C. After 24 hours the inocula were subjected to serial dilution. For each sample 10 duly marked test tubes were used. In the first tube 9 ml. of sterile saline solution (Normal Saline 0.9% NaCl) was added and in rest of the tubes 10 ml. of normal saline solution was added. 1 ml. of enriched sample from the incubated broth was taken and added to the 1^{st} tube. Subsequently 1 ml. was collected from the first tube and transferred to the second tube. Similarly the procedure was repeated until 10-fold dilutions (from 10^{-1} to 10^{-10}) was achieved. After the serial dilution all of the tubes were incubated at 37^0 c for 24 hrs. 0.1 ml (100 µl.) of the inoculum from 8^{th} and 10^{th} dilution tubes were streaked across respective MHA plates using a sterile bacteriological loop and further incubated at 37^0C for 24 hours. After 24 hours the colony morphology was studied and bacterial load was enumerated using a digital colony counter . Colonies ranging from 30-300 µ in size were taken into consideration for bacterial load estimation and no. of colonies were subsequently recorded.

In the similar manner the faecal sample was mixed with sterile normal saline solution and volume was adjusted to 10 mL . Each pool was diluted serially via 10-fold dilutions using afore mentioned procedure.

3.4 Isolation of eco-friendly micro-organisms from intestine of chicks:

In the present study various beneficial micro-organisms were isolated using specific culture media in compliance with routine microbiological procedure. The synthetic dehydrated culture media were obtained from Hi Media, Mumbai and manufacturer's instructions were followed.

In order to isolate *Lactobacillus* species the samples were inoculated in primary enriched media i.e. MRS broth at 37^0C for 24 hours. The inocula were spreaded across Rogosa agar plates in order to obtain pure culture. Sample diagnostic for pure culture was stored for further bacteriological analysis.

In order to isolate *Bacteroides* species the samples were inoculated in primary enriched media i.e. BHI broth at 37^0C for 24 hours. The inocula were spreaded across Wilkins Chalgren agar plates to obtain pure culture. Sample diagnostic for pure culture was stored for further bacteriological analysis.

Primary culture media i.e. BSM broth was employed for enrichment of *Bifidobacterium* species which was incubated at 37^0C for 24 hours. The inocula were spreaded across BSM agar paltes to obtain pure culture. Sample diagnostic procedure was stored for further bacteriological analysis.

Primary culture media i.e. thioglycolate broth supplemented with 0.5 ml of peptic digest of blood (BBL), vitamin K_I, and hemin were used for enrichment of *Clostridium* species and they were subsequently incubated at 37^0C for 24 hours. The inocula were spreaded across CCFA agar plates to obtain pure culture which were stored for future bacteriological analysis.

BHI broth serves the role of primary enrichment media for *Enterococcus* species. The inocula from the incubated samples were spreaded across BEA agar to obtain pure culture. Sample diagnostic for pure culture was stored for further bacteriological analysis.

In order to isolate *Corynebacterium* species the samples were inoculated in primary enriched media i.e. BHI broth in an atmosphere of 5% CO_2 at 37^0C for 24 hours. The inocula were spreaded across Hoyle′s tellurite agar plates in order to obtain pure culture. Sample diagnostic for pure culture was stored for further bacteriological analysis.

3.5 Biofilm formation assay:

3.5.1 Congo red agar (CRA) method:

For assessment of biofilm formation Freeman *et al.,* (1989) method was followed with slight modification. The medium was composed of Brain Heart Infusion (BHI) agar(Hi Media,Mumbai) 40 g/l, sucrose 50g/l and Congo red dye(Hi Media, Mumbai) 0.8g/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was added when the agar had cooled to 55°C. Plates of the medium were inoculated and incubated aerobically for 24 hours at 37°C. Black colonies with a dry crystalline consistency indicated positive result. Non slime producers usually remains pink, though occasional darkening at the centre of colony may be observed.

3.5.2 Tube method:

The qualitative method of biofilm formation was performed according to the method described by Dheepa *et al*. (2011) with a little modification. Glass tubes filled with 3ml of BHI broth (Hi Media,Mumbai) and 1% sucrose were inoculated with a loopful of a pure culture. After 48hr of incubation at 37^0 C, the content of each tube was decanted. The tubes were then stained with 1% saffranin for 7mins. Then the tubes were washed with distilled water for 5mins. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube. Presence of stained material at the liquid air interface alone was not regarded as indicative of slime production.

3.6 Study of Antibiotic Sensitivity Test profile of eco-friendly gut microflora:

The antimicrobial susceptibility testing was determined against 9 antimicrobial drugs using Kirby-Bauer disc diffusion methods. The test was carried out according to NCCLS (National Committee On Clinical Laboratory Standards) protocols (CLSI-2009). The diameter of zone of inhibition was measured, recorded, and interpreted in accordance with NCCLS guidelines . Mueller Hinton agar (MHA) plates were swabbed with test cultures using a sterile cotton swab. These plates were allowed to dry for 5 minutes. Then different antibiotic discs were placed manually using a sterile

fine forcep. The plates were incubated at 37^0C for 24 hours under micro aerobic condition for antibiotic sensitivity. All the antibiotic discs were obtained from HIMEDIA, Mumbai. Various anti microbial discs were employed such as Vancomycin (30mg), Gentamicin (30mg), Tobramycin (10mg), Tetracycline (30mg), Azithromycin (30mg), Ampicillin (2mg), Kanamycin (30mg), Nalidixic Acid (30mg) and Metronidazole (5mg).

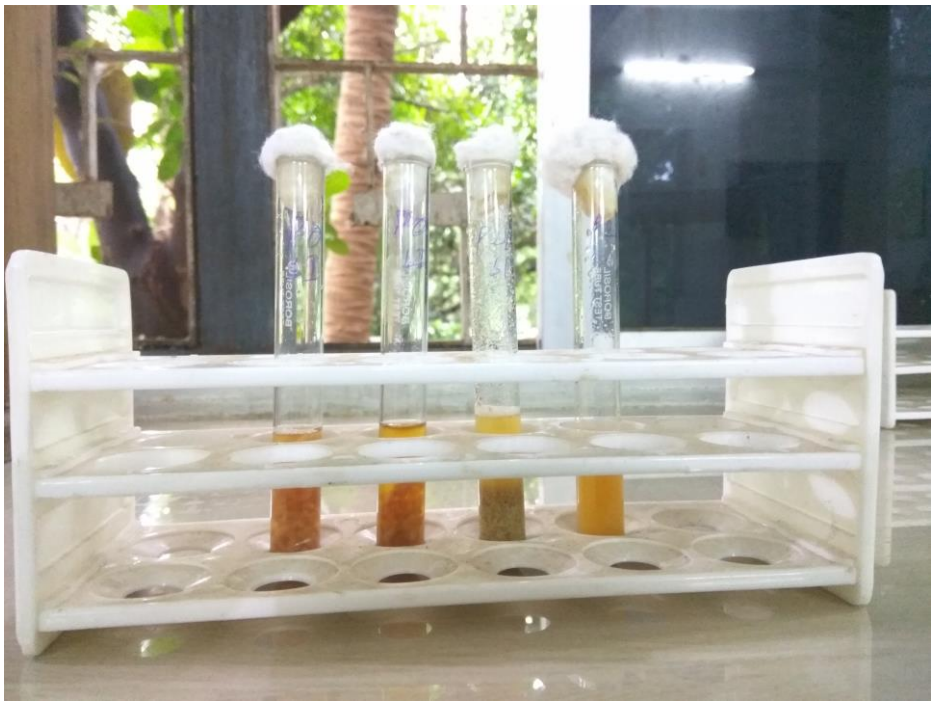


Fig 3.1 Primary enrichment of intestinal sample in BHI broth

Fig 3.2 Procedure of serial dilution

RESULTS

Eco-friendly microbes in the gastro intestinal tract of chicks help in digestion of food and detoxification of certain harmful compounds. Besides that they play crucial roles in the health and immune system of chickens. The microbial community present in different parts of intestine were same but the load was found to be different .Administration of these eco-friendly microbes to chicks in the form of probiotics will help in attaining sustained growth rate.

In the present study a total no. of (n=425) samples consisting of both intestinal and faecal samples were collected from various breeds in weekly interval. Then they were subjected to routine isolation and identification procedure as per the method of Cruickshank et al. which revealed various types of microbes present in the chicken intestine with their load. Besides that microscopic analysis and biochemical characterization were performed.

4.1 Percentage of prevalence of various eco-friendly microbes in different parts of chick intestine

Total no. of 308 intestinal samples which consist of Jejunum(n=81), Ileum(n=75), Caecum(n=79), Colon(n=73) were subjected to routine bacteriological procedure for estimation of prevalence of various eco-friendly microbes in different parts of the chick intestine.

From total 81 no. of jejunum samples 48 samples showed predominance of *Lactobacillus* species indicating 59.3 % of prevalence. Similarly in 11 samples *Clostridium* species was found to be abundant (13.5 %). 2 and 6 no. of jejunum samples showed predominance of *Bifidobacterium* and *Enterococcus* species indicating 2.5 % and 7.5% prevalence respectively. Out of 81 samples prevalence of *Corynebacterium* species was found to be 6.1% (5 out of 81). *Bacteroides* species showed minimum prevalence i.e. 2.4% (2 samples out of 81).

Total no. of 75 ileum samples were observed out of which in 53 samples *Lactobacillus* species was predominant which indicated a prevalence of 70.8 %. In 8 ileum samples *Clostridium* species was abundant showing the prevalence of 10.7%. 3 samples out of 75 ileum samples showed abundance of *Bifidobacterium* species

(4% prevalence) and in 3 samples *Enterococcus* species was abundant (4% prevalence). In 2 samples *Corynebacterium* species was found to be abundant(2.6% prevalence) and *Bacteroides* species again showed least abundance (1.3% prevalence).

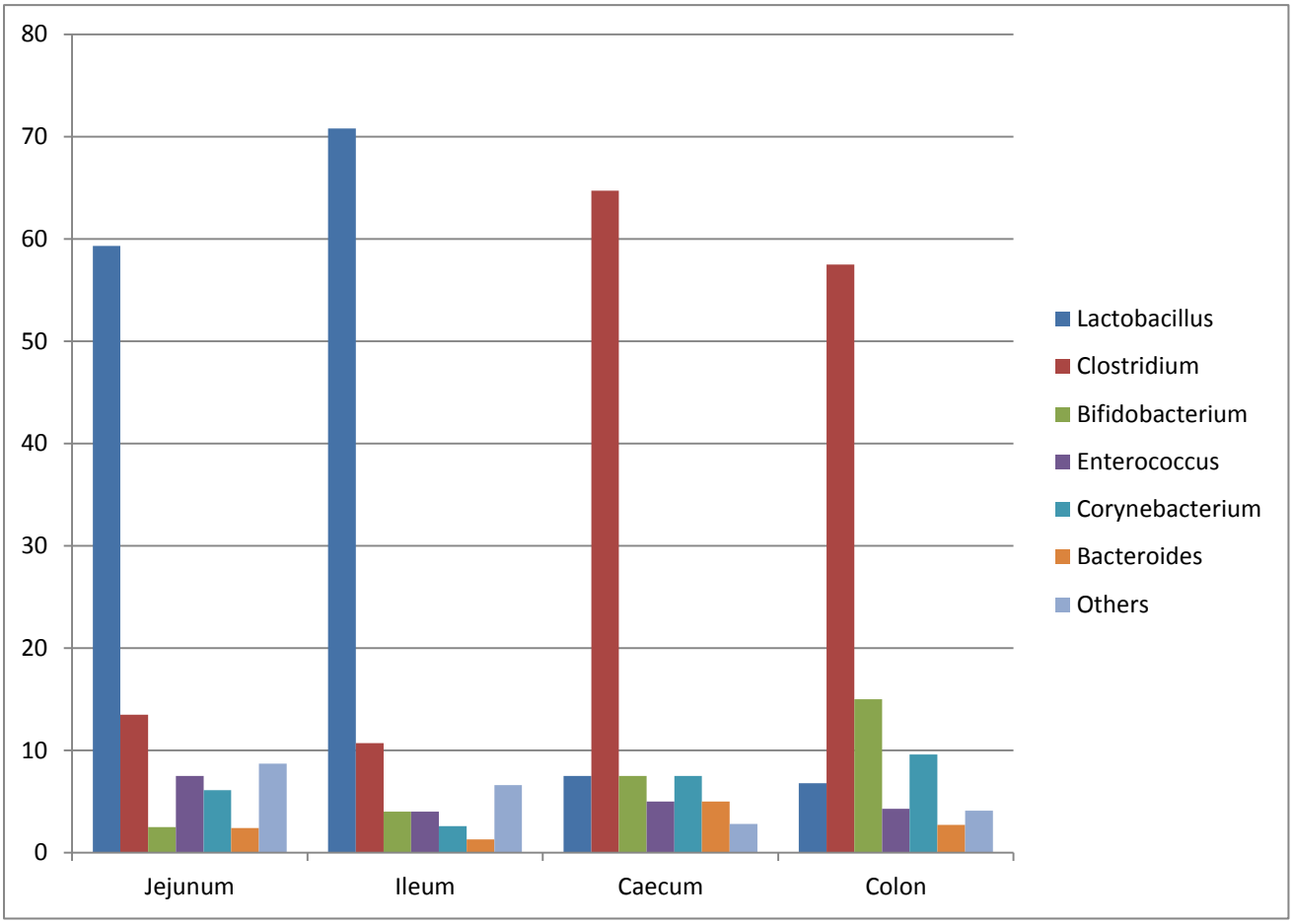
But the observation of 79 caecal samples showed a different trend in terms of abundance of eco-friendly microbes. In caecum *Clostridium* species was found to be the most abundant bacteria showing 64.7% of prevalence (51 out of 79 samples).*Lactobacillus* species was abundant in 6 samples (7.5% abundance) and same followed for *Bifidobacterium* and *Corynebacterium* species too. *Enterococcus* and *Bacteroides* species were predominant in 4 samples each indicating a prevalence of 5%.

A total no. of 73 colonic samples were processed for routine microbiological observation in which *Clostridium* species emerged predominant with a prevalence of 57.5% (42 samples out of 73). 11 samples showed predominance of *Bifidobacterium* species while 7 samples showed abundance of *Corynebacterium* species. In 5 samples *Lactobacillus* species was abundant while in 3 samples *Enterococcus* species was predominant. In only 2 samples *Bacteroides* species was predominant indicating the lowest prevalence of 2.7% in colon.

Table 4.1 depicts percentage of prevalence of various eco-friendly microbes in different parts of chick intestine

Name of the species isolated	Total number of intestinal samples(n=308)							
	No. of samples of Jejunum(n=81)		No. of samples of Ileum(n=75)		No. of samples of Caecum(n=79)		No. of samples of Colon(n=73)	
	No. of isolates	% of prevalence	No. of isolates	% of prevalence	No. of isolates	% of prevalence	No. of isolates	% of prevalence
Lactobacillus species	48/81	59.3	53/75	70.8	6/79	7.5	5/73	6.8
Clostridium species	11/81	13.5	8/75	10.7	51/79	64.7	42/73	57.5
Bifidobacterium species	2/81	2.5	3/75	4	6/79	7.5	11/73	15
Enterococcus species	6/81	7.5	3/75	4	4/79	5	3/73	4.3
Corynebacterium species	5/81	6.1	2/75	2.6	6/79	7.5	7/73	9.6
Bacteroides species	2/81	2.4	1/75	1.3	4/79	5	2/73	2.7
Others	7/81	8.7	5/75	6.6	2/79	2.8	3/73	4.1

Fig. 4.1 shows percentage of prevalence of various eco-friendly microbes in different parts of chick intestine



4.2 Percentage of appearance different eco-friendly microbes in chicken intestine in various stages of growth:

The below mentioned table revealed that 16 no. of samples were collected from day old birds out of which 6 samples showed prevalence of *Clostridium* species (37.5%) where *Lactobacillus* species was found to be 25% prevalent. *Bacteroides* species showed 12.5% prevalence while *Bifidobacterium*, *Enterococcus*,and *Corynebacterium* species each showed 6.25% prevalence.

Similarly, 16 no. of samples were collected from 1 week age group of birds out of which 7 samples showed prevalence of *Clostridium* species (43.75%) where *Lactobacillus* species was found to be 18.75% prevalent. *Corynebacterium* species showed 12.5% prevalence while *Bifidobacterium* species, *Enterococcus* species and *Bacteroides* species each showed 6.25% prevalence.

19 no. of samples were collected from 2 week age group of birds out of which 6 samples showed prevalence of *Clostridium* species (31.6%) where *Lactobacillus* species was found to be 15.8% prevalent. *Bifidobacterium* species showed 21.1% prevalence while *Enterococcus* species and *Bacteroides* species each showed 10.5% prevalence. *Corynebacterium* showed minimum prevalence of 5.25%.

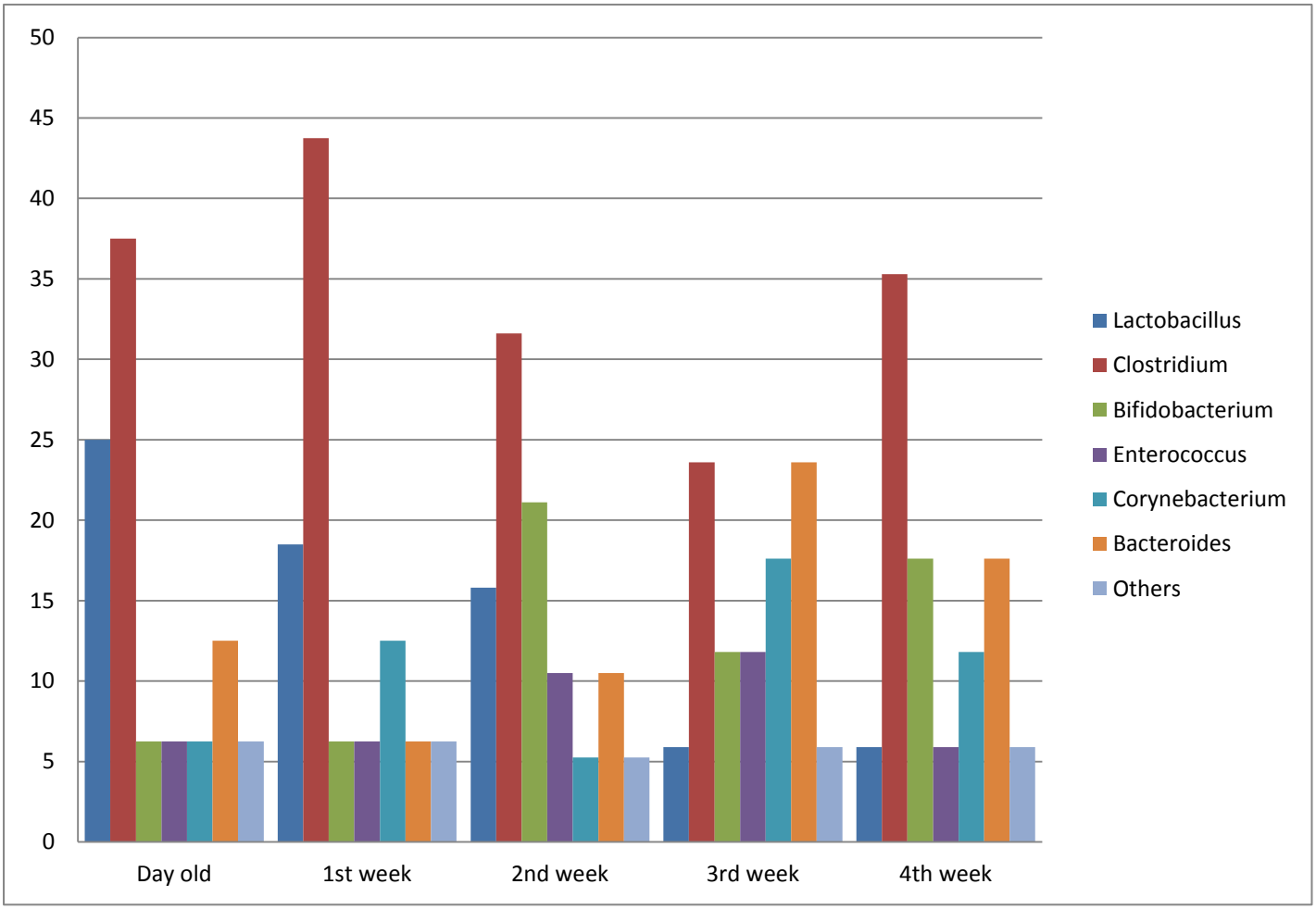
17 no. of samples were collected from 3 week age group of birds out of which 4 samples showed prevalence of *Clostridium* species (23.6%) where *Lactobacillus* species was found to be 5.9% prevalent. *Corynebacterium* species showed 17.6% prevalence while *Enterococcus* and *Bifidobacterium* species each showed 11.8% prevalence. *Bacteroides* showed 23.6% prevalence.

17 no. of samples were collected from 4 week age group of birds out of which 6 samples showed prevalence of *Clostridium* species (35.3%) where *Lactobacillus* species was found to be 5.9% prevalent. *Corynebacterium* species showed 11.8% prevalence while *Bacteroides* and *Bifidobacterium* species each showed 17.6% prevalence. *Enterococcus* species showed 5.9% prevalence.

Table 4.2 depicts percentage of appearance different eco-friendly microbes in chicken intestine in various stages of growth

Name of the species isolated	No. of weeks									
	Day old(n=16)		1st week(n=16)		2nd week(n=19)		3rd week(n=17)		4th week(n=17)	
	No. of isolates	% of prevalence	No. of isolates	% of prevalence	No. of isolates	% of prevalence	No. of isolates	% of prevalence	No. of isolates	% of prevalence
Lactobacillus species	4/16	25	3/16	18.75	3/19	15.8	1/17	5.9	1/17	5.9
Clostridium species	6/16	37.5	7/16	43.75	6/19	31.6	4/17	23.6	6/17	35.3
Bifidobacterium species	1/16	6.25	1/16	6.25	4/19	21.1	2/17	11.8	3/17	17.6
Enterococcus species	1/16	6.25	1/16	6.25	2/19	10.5	2/17	11.8	1/17	5.9
Corynebacterium species	1/16	6.25	2/16	12.5	1/19	5.25	3/17	17.6	2/17	11.8
Bacteroides species	2/16	12.5	1/16	6.25	2/19	10.5	4/17	23.6	3/17	17.6
Others	1/16	6.25	1/16	6.25	1/19	5.25	1/17	5.9	1/17	5.9

Fig 4.2 shows percentage of appearance different eco-friendly microbes in chicken intestine in various stages of growth



4.3 Biochemical characteristics of isolated eco-friendly gut microflora:

The table mentioned below shows the biochemical characteristics of various eco-friendly bacteria. *Lactobacillus* (Gram+ve) fermented glucose, fructose, lactose, maltose, arabinose and didn't ferment mannose. It produced gas on carbohydrate fermentation and didn't produce H_2S. Besides that it showed negative catalase and oxidase activity. Similary *Bifidobacterium*, a gram+ve bacteria fermented glucose, fructose, lactose, maltose and mannose while it didn't ferment arabinose. It produced neither gas nor H_2S.It also showed negative catalase and oxidase activity. *Corynebacterium*, a gram+ve bacteria fermented glucose, fructose, lactose and maltose. It produced gas on glucose fermentation but didn't produce H_2S. It showed positive catalase activity and negative oxidase activity. *Clostridium*, the predominant bacteria of large intestine is a gram+ve one. It fermented glucose, fructose and mannose while it couldn't ferment lactose, maltose and arabinose. It produced both gas and H_2S. It showed neither catalase activity nor oxidase activity. *Enterococcus* , a gram+ve bacteria fermented glucose, fructose, lactose, maltose and mannose except arabinose. It didn't produce H_2S. Besides that it showed negative catalase as well as negative oxidase activity. *Bacteroides*, a gram-ve bacteria fermented glucose, lactose, maltose and didn't ferment fructose, mannose and arabinose. It didn't produce gas on carbohydrate fermentation while it could produce H_2S. It also showed both catalase and oxidase activity. Hereby it is concluded that all the eco-friendly bacteria isolated from the chicken intestine are glucose fermenters.

Table 4.3 depicts biochemical characteristics of isolated eco-friendly gut microflora

		Biochemical profiling										
Name of the species isolated	Gram staining	Glucose	Fructose	Lactose	Maltose	Mannose	Arabinose	Catalase	Coagulase	Oxidase	Gas	H_2S
Lactobacillus species	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-	-ve	+ve	-ve
Bifidobacterium species	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-	-ve	-ve	-ve
Corynebacterium species	+ve	+ve	+ve	+ve	+ve	-	-ve	+ve	-	-ve	+ve	-ve
Clostridium species	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-	-ve	+ve	+ve
Enterococcus species	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-	-ve	-	-ve
Bacteroides species	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-	+ve	-ve	+ve

4.4 Microbial load present in various intestinal parts of different breeds of chicks at various stages of growth (in cfu/ml.):

The microbial load of various parts of the intestine such as jejunum, ileum, caecum and colon of four different chick breeds at various stages of growth were enumerated and mentioned in the table below.

In jejunum of day old Kalinga brown chicks the microbial load was found to be 1.5×10^9 cfu/ml. while in ileum the load was 1×10^9 cfu/ml. In caecum the microbial load was 1.7×10^{10} cfu/ml. and in colon the load was 1.2×10^9 cfu/ml.. Similarly in 1st week the microbial load in jejunum, ileum, caecum and colon were 1.7×10^9, 1.5×10^9 ,2.1×10^{10}, 2.3×10^9 cfu/ml. respectively. In 2nd week the load estimated in jejunum, ileum, caecum and colon were found to be 2.3×10^9, 1.9×10^9, 2.4×10^{10}, 1.9×10^9 cfu/ml. respectively. In 3rd week the microbial load in jejunum, ileum, caecum and colon were 2.7×10^9, 2.8×10^9, 2.9×10^{10}, 2.9×10^9 cfu/ml. respectively. In 4th week the microbial load in jejunum, ileum, caecum and colon were 3.3×10^9, 3.7×10^9, 4.1×10^{10}, 3.2×10^9cfu/ml. respectively.

In the similar fashion the microbial load in different intestinal parts of Colour Cross breed at subsequent stages of growth was estimated. In day old Colour Cross chick the microbial load in jejunum, ileum, caecum and colon was found to be 1.3×10^9, 1.2×10^9, 1.4×10^{10}, 1×10^9 cfu/ml. respectively. In 1st week of age the microbial load in jejunum, ileum, caecum and colon was 1.8×10^9, 1.7×10^9, 1.8×10^{10}, 1.6×10^9 cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was 2.1×10^9, 2.2×10^9, 2.5×10^{10}, 2.3×10^9 cfu/ml. respectively. In 3rd weeks of growth stage the microbial load in jejunum, ileum, caecum and colon was found to be 2.6×10^9, 2.5×10^9, 2.7×10^{10}, 2.8×10^9 cfu/ml. respectively. In 4thweek the microbial load in jejunum, ileum, caecum and colon was found to be 3.2×10^9, 2.8×10^9, 3.9×10^{10}, 3.5×10^9 cfu/ml. respectively.

In day old chicks of Banaraja the microbial load in jejunum, ileum, caecum and colon was 1.1×10^9, 1×10^9, 1.4×10^{10}, 1.3×10^9 cfu/ml. respectively. In 1st week of age the microbial load in jejunum, ileum, caecum and colon was 1.7×10^9, 1.3×10^9, 1.9×10^{10}, 1.6×10^9 cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 1.5×10^9, 2.1×10^9, 2.2×10^{10}, 2.6×10^9cfu/ml.

respectively. In 3rd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 2.2×10^9, 2.9×10^9, 3.1×10^{10}, 1.5×10^9cfu/ml. respectively. In 4th week of age microbial load in jejunum, ileum, caecum and colon was 2.5×10^9, 3.8×10^9, 4.2×10^{10}, 3.5×10^9cfu/ml. respectively.

Lastly the microbial load in different intestinal parts of Hubbard breed at subsequent stages of growth was enumerated. In day old chicks the microbial load in jejunum, ileum, caecum and colon was 1.2×10^9, 1.3×10^9, 1.5×10^{10}, 1.4×10^9cfu/ml. respectively. In 1st week the microbial load in jejunum, ileum, caecum and colon was 1.7×10^9, 1.8×10^9, 1.9×10^{10} 2.1×10^9cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 2.3×10^9, 2.7×10^9, 2.8×10^{10},2.5×10^9cfu/ml. respectively. In 3rd week of age the microbial load in jejunum, ileum, caecum and colon was found to be 2.5×10^9, 2.1×10^9, 3.2×10^{10}, 3.4×10^9cfu/ml. respectively. In 4th week of age the microbial load in jejunum, ileum, caecum and colon was 2.8×10^9, 3.1×10^9, 4.5×10^{10}, 3.6×10^9 cfu/ml. respectively.

By analysing the results it was concluded that the average microbial load in jejunum was found to be highest in Kalinga Brown breed. Similary when the average microbial load of ileal contents were analysed ,it was observed that in Banaraja breed the microbial load in ileum was found to be maximum. In the similar fashion the average microbial load in caecum and colon was maximum in Hubbard breed.

Table 4.4 depicts microbial load present in various intestinal parts of different breeds of chicks at various stages of growth (in cfu/ml.)

	Name of the breed															
	Kalinga Brown				Colour Cross				Banaraja				Hubbard			
No. of week	Jejunum	Ileum	Caecum	Colon	Jejunum	Ileum	Caecum	Colon	Jejunum	Ileum	Caecum	Colon	Jejunum	Ileum	Caecum	Colon
Day old	1.5×10^{9}	1×10^{9}	1.7×10^{10}	1.2×10^{9}	1.3×10^{9}	1.2×10^{9}	1.4×10^{10}	1×10^{9}	1.1×10^{9}	1×10^{9}	1.4×10^{10}	1.3×10^{9}	1.2×10^{9}	1.3×10^{9}	1.5×10^{10}	1.4×10^{9}
1	1.7×10^{9}	1.5×10^{9}	2.1×10^{10}	2.3×10^{9}	1.8×10^{9}	1.7×10^{9}	1.8×10^{10}	1.6×10^{9}	1.7×10^{9}	1.3×10^{9}	1.9×10^{10}	1.6×10^{9}	1.7×10^{9}	1.8×10^{9}	1.9×10^{10}	2.1×10^{9}
2	2.3×10^{9}	1.9×10^{9}	2.4×10^{10}	1.9×10^{9}	2.1×10^{9}	2.2×10^{9}	2.5×10^{10}	2.3×10^{9}	1.5×10^{9}	2.1×10^{9}	2.2×10^{10}	2.6×10^{9}	2.3×10^{9}	2.7×10^{9}	2.8×10^{10}	2.5×10^{9}
3	2.7×10^{9}	2.8×10^{9}	2.9×10^{10}	2.9×10^{9}	2.6×10^{9}	2.5×10^{9}	2.7×10^{10}	2.8×10^{9}	2.2×10^{9}	2.9×10^{9}	3.1×10^{10}	1.5×10^{9}	2.5×10^{9}	2.1×10^{9}	3.2×10^{10}	3.4×10^{9}
4	3.3×10^{9}	3.7×10^{9}	4.1×10^{10}	3.2×10^{9}	3.2×10^{9}	2.8×10^{9}	3.9×10^{10}	3.5×10^{9}	2.5×10^{9}	3.8×10^{9}	4.2×10^{10}	3.5×10^{9}	2.8×10^{9}	3.1×10^{9}	4.5×10^{10}	3.6×10^{9}

Fig 4.3 shows microbial load present in various intestinal parts of different breeds of chicks at day old age

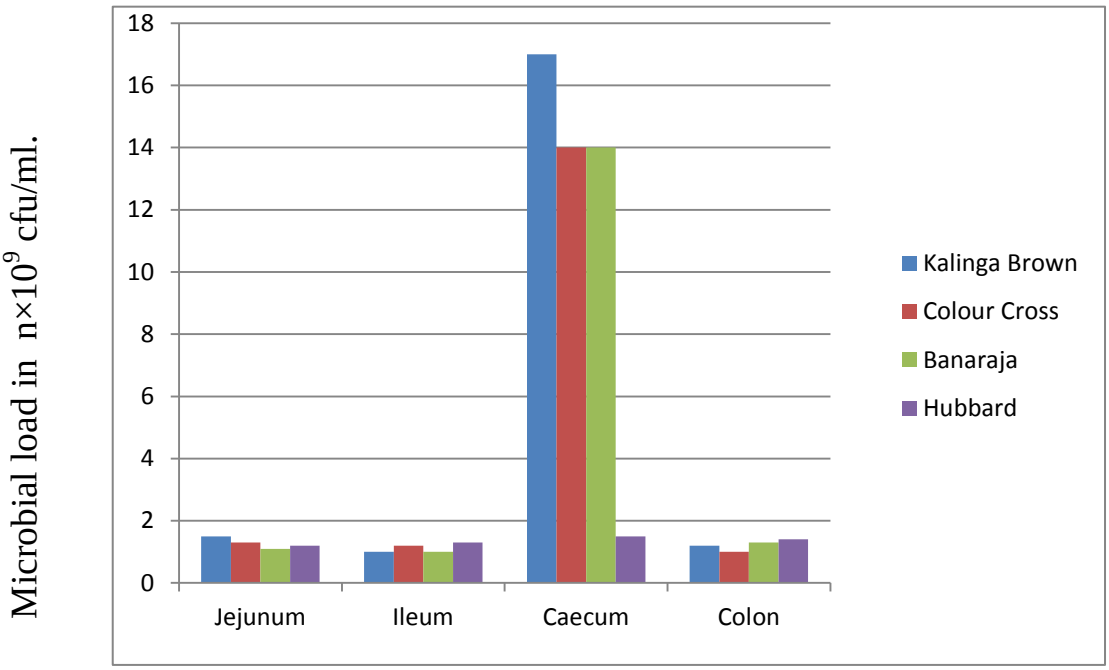


Fig 4.4 shows microbial load present in various intestinal parts of different breeds of chicks at 1st week of age

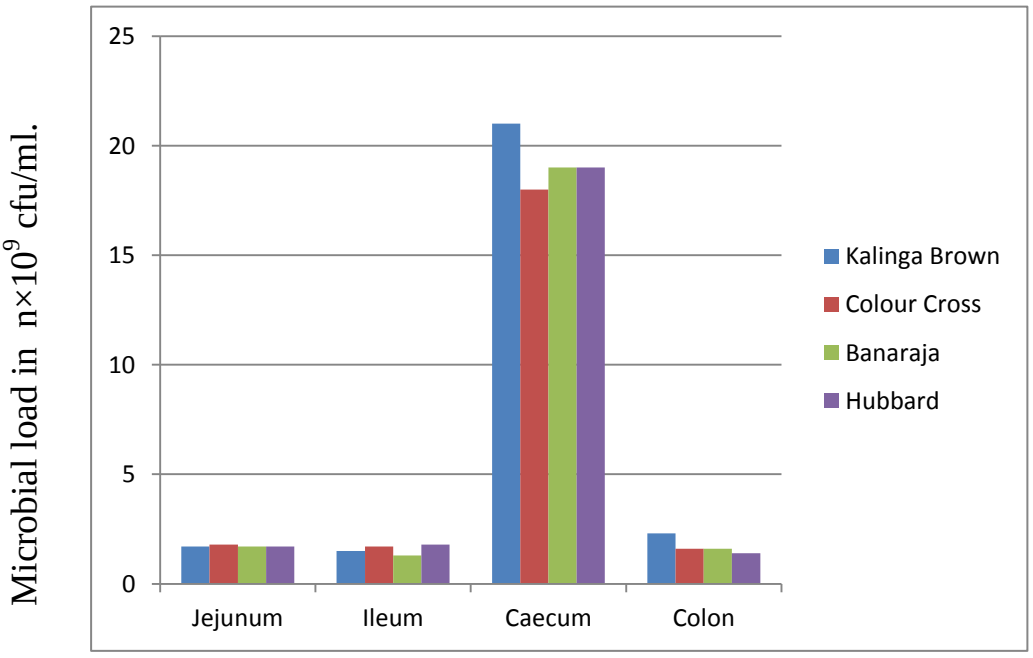


Fig. 4.5 shows microbial load present in various intestinal parts of different breeds of chicks at 2nd week of age

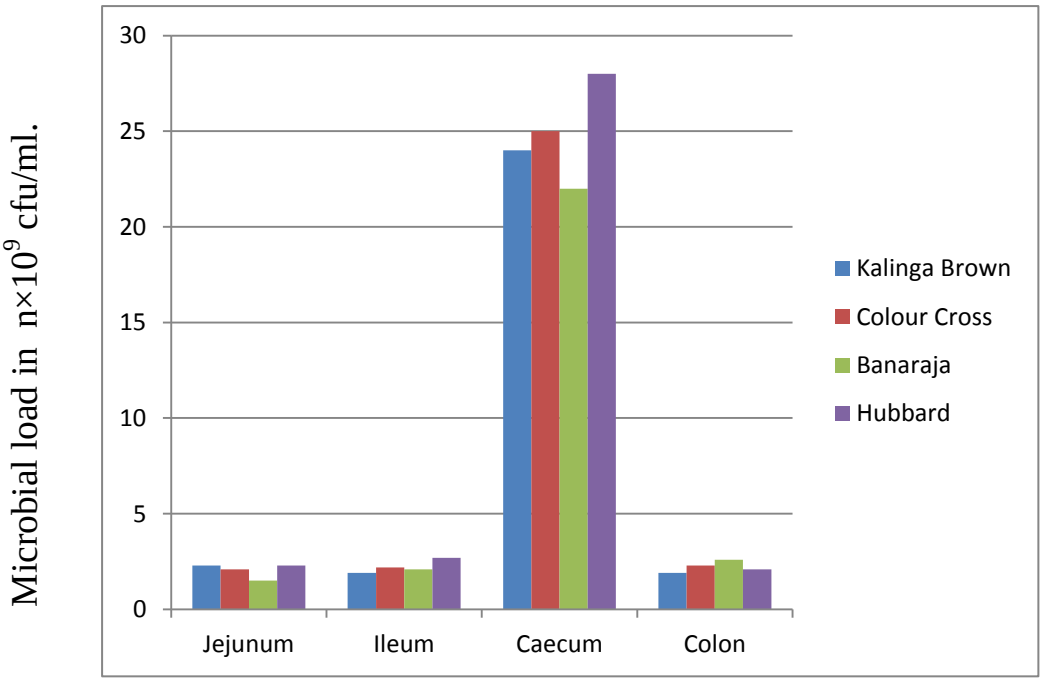


Fig . 4.6 shows microbial load present in various intestinal parts of different breeds of chicks at 3rd week of age

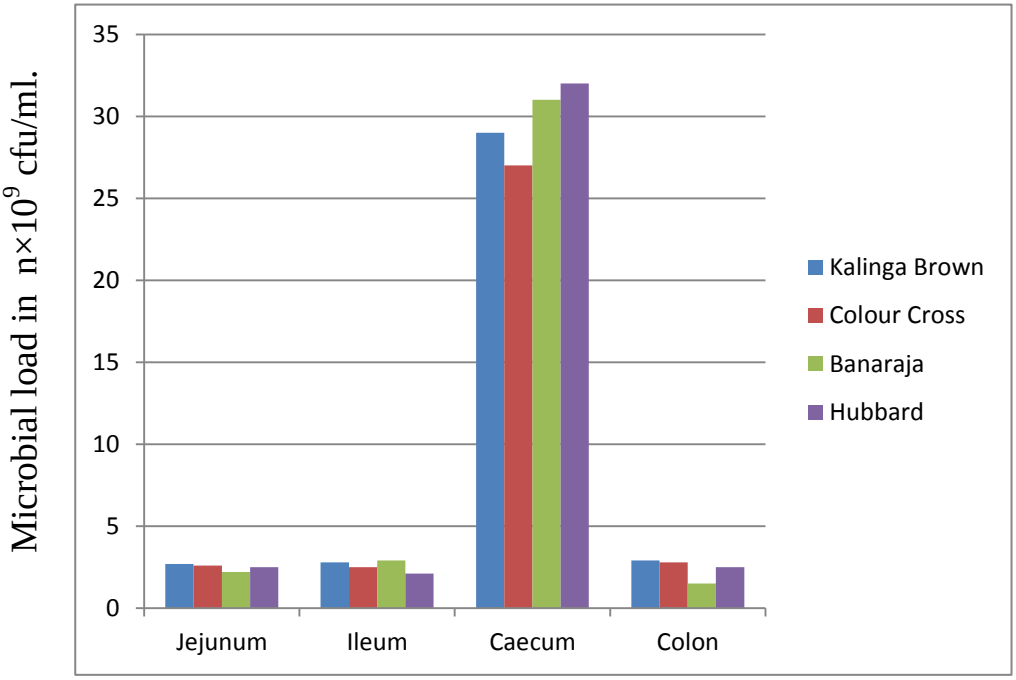
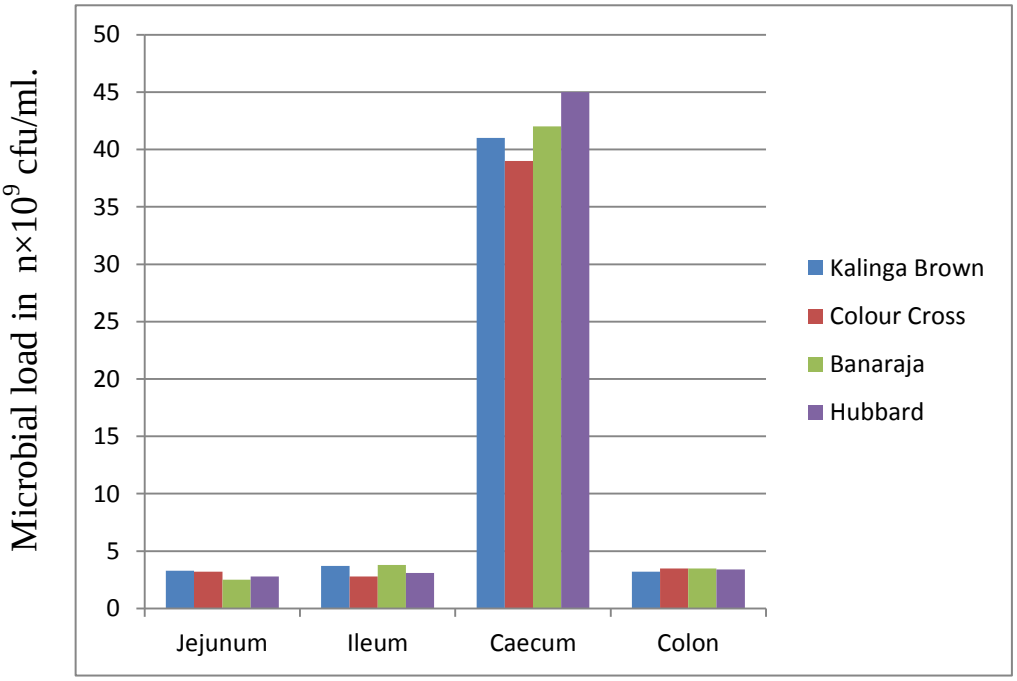


Fig . 4.7 shows microbial load present in various intestinal parts of different breeds of chicks at 4th week of age



4.5 Microbial load in faeces of different breeds of chicks at various stages of growth:

Total 85 no. of faecal samples were collected from four different breeds at different stages of growth in weekly interval and the average microbial load in the faeces were recorded.

From the below mentioned table it was revealed that 23 faecal samples of Kalinga Brown breed were analysed which consisted of 4 samples from day old chicks, 4 samples from 1st week , 4 samples from 2nd week, 5 samples from 3rd week and 6 samples from 4th week. The average microbial load in day 0,1st week, 2nd week, 3rd week and 4th week was found to be 3.4×10^5, 4.1×10^5, 5.4×10^5, 6.7×10^5, 7.9×10^5 cfu/ml. respectively.

Total 21 faecal samples from Colour Cross breeds were observed which consisted of 4 samples from day old chicks, 4 samples from 1st week , 4 samples from 2nd week, 4 samples from 3rd week and 5 samples from 4th week of growth stage.The average microbial load in day 0,1st week, 2nd week, 3rd week and 4th week was found to be 2.5×10^5, 3.4×10^5, 4.1×10^5, 5.3×10^5, 6.9×10^5 cfu/ml. respectively.

17 faecal samples of Banaraja breed were analysed from which 3 samples were from day old chicks, 4 samples were from 1st week , 4 sampleswere from 2nd week, 3 samples were from 3rd week and 3 samples were from 4th week.The average microbial load in day 0,1st week, 2nd week, 3rd week and 4th week was found to be 3.1×10^5, 4.3×10^5, 4.8×10^5, 5.7×10^5, 6.3×10^5 cfu/ml. respectively.

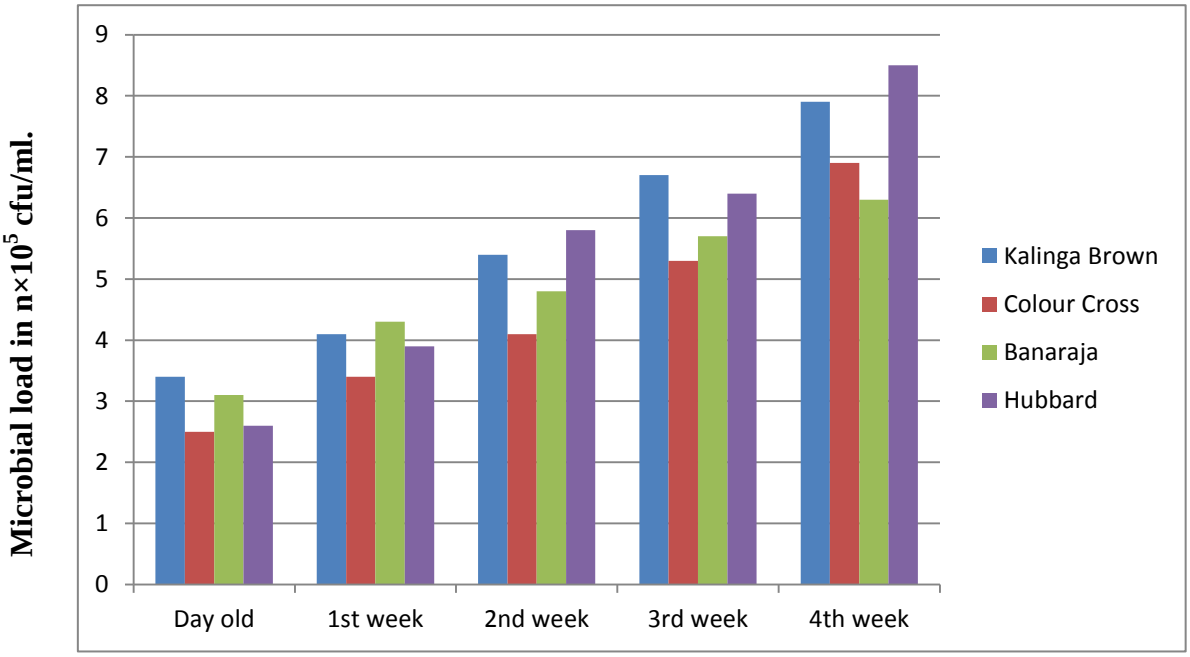
24 faecal samples of Hubbard breed were analysed from which 5 samples were from day old chicks, 4 samples were from 1st week , 7 samples were from 2nd week, 5 samples were from 3rd week and 3 samples were from 4th week.The average microbial load in day 0,1st week, 2nd week, 3rd week and 4th week was estimated to be 2.6×10^5, 3.9×10^5, 5.8×10^5, 6.4×10^5, 8.5×10^5 cfu/ml. respectively.

By observing the average microbial load in faeces obtained at various stages of growth in different breeds it was concluded that at day old age maximum microbial load was found in faeces of Kalinga Brown breed. At 1^{st} week of age the average microbial load was maximum in faeces of Banaraja breed. At 2^{nd} week of age the average microbial load was found to be highest in faeces of Hubbard breed. At 3^{rd} week of age the average microbial load was highest in faeces of Kalinga Brown breed. At 4^{th} week of age the highest average microbial load was found in faeces of Hubbard breed.

Table 4.5 depicts microbial load in faeces of different breeds of chicks at various stages of growth

Name of the breed	Age in weeks	Microbial load in cfu/ml.
Kalinga Brown(n=23)	Day old(n=4)	3.4×10^5
	1(n=4)	4.1×10^5
	2(n=4)	5.4×10^5
	3(n=5)	6.7×10^5
	4(n=6)	7.9×10^5
Colour Cross(n=21)	Day old(n=4)	2.5×10^5
	1(n=4)	3.4×10^5
	2(n=4)	4.1×10^5
	3(n=4)	5.3×10^5
	4(n=5)	6.9×10^5
Banaraja(n=17)	Day old(n=3)	3.1×10^5
	1(n=4)	4.3×10^5
	2(n=4)	4.8×10^5
	3(n=3)	5.7×10^5
	4(n=3)	6.3×10^5
Hubbard(n=24)	Day old(n=5)	2.6×10^5
	1(n=4)	3.9×10^5
	2(n=7)	5.8×10^5
	3(n=5)	6.4×10^5
	4(n=3)	8.5×10^5

Fig. 4.8 shows microbial load in faeces of different breeds of chicks at various stages of growth



4.6 Assay of Biofilm formation by eco-friendly microflora:

4.6.1 CRA method of biofilm detection:

In CRA Method, the isolates showing black coloured colonies with a dry crystalline consistency were considered as positive, isolates producing pink colonies considered negative and dark colonies with partial colouration without a dry crystalline consistency were taken as weak for biofilm production. This method showed that from 20 isolates of *Lactobacillus*, 10 (50%) showed negative, 4 (20%) moderate and 6 (30%) positive for biofilm formation. The results are summarised in table 4.6.1.

Table 4.6.1. Shows detection of biofilm formation by CRA method:

Sl No	Total isolates	Negative		Moderate		Positive	
		No	%	No	%	No	%
1	20	10	50	4	20	6	30

4.6.2. Detection of biofilm formation by Tube method:

In this method, positive results were indicated by presence of an adherent film of stained material on the inner surface of the tubes and were classified as no adherent (0), weakly adherent (+), moderately adherent (+ +) and strongly adherent (+ + +) as per the extent of adherence of the stain. The summary is presented in table 4.6.2. As per the table it was revealed that from 20 isolates of *Lactobacillus*, 7 (35%), 1 (5%), 6 (30%), 6 (30%) showed no, weak, moderate and strong adherence respectively.

Table 4.6.2. Shows detection of biofilm formation by tube method:

Sl.no	Total isolate	No adherence		Weak adherence		Moderate adherence		Strong adherence	
		No	%	No	%	No	%	No	%
1	20	7	35	1	5	6	30	6	30

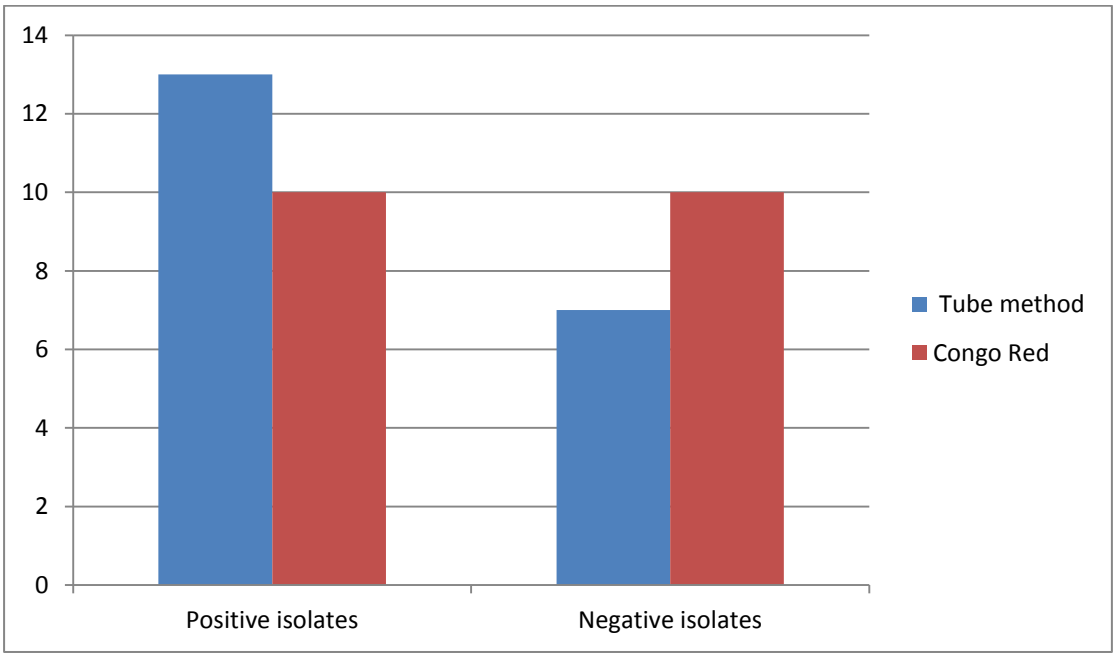
4.6.3 Comparative study of Tube method and Congo red method for detection of biofilm:

In this present comparative study of the above two biofilm detection methods it was found that out of a total 20 no. of isolates of *Lactobacillus* , 13 isolates (65%) showed positive for biofilm formation in tube method and 10 isolates (50%) showed positive for biofilm formation in congo red method.

Table. 4.6.3 Comparative study of Tube method and Congo red method for detection of biofilm formation by *Lactobacillus*:

Sl. No	No of isolates	Tube method		Congo Red method	
		Biofilm positive (no)	Biofilm positive (%)	Biofilm positive (no)	Biofilm positive (%)
1	20	13	65	10	50

Fig. 4.9 shows comparative study of Tube method and Congo red method for detection of biofilm formation by Lactobacillus



4.7 Antibiotic Sensitivity Test profile of eco-friendly gut microflora:

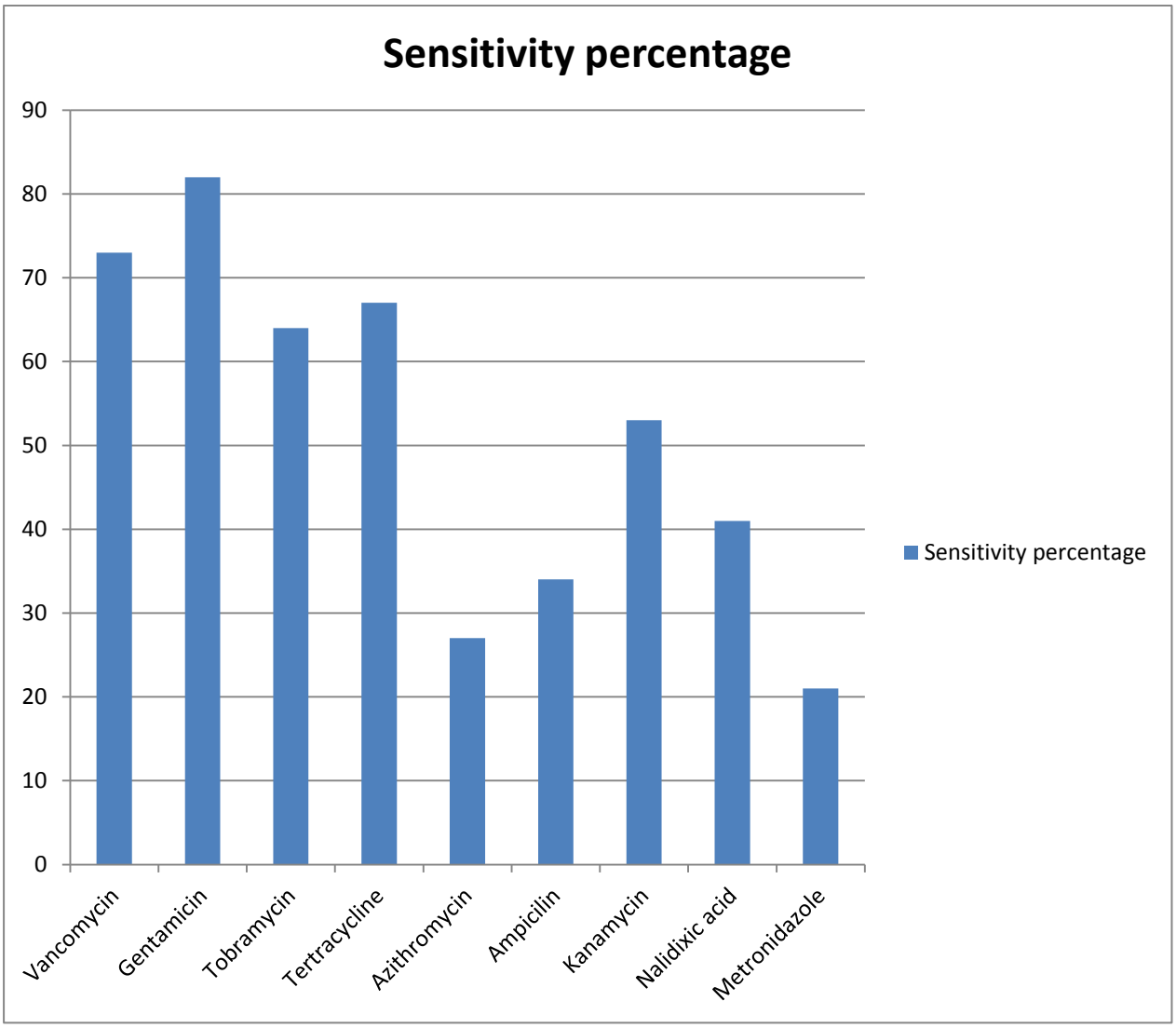
In the present study 57 intestinal samples from different breeds of chicks were subjected to antibiotic sensitivity profile in different media and the results were recorded.

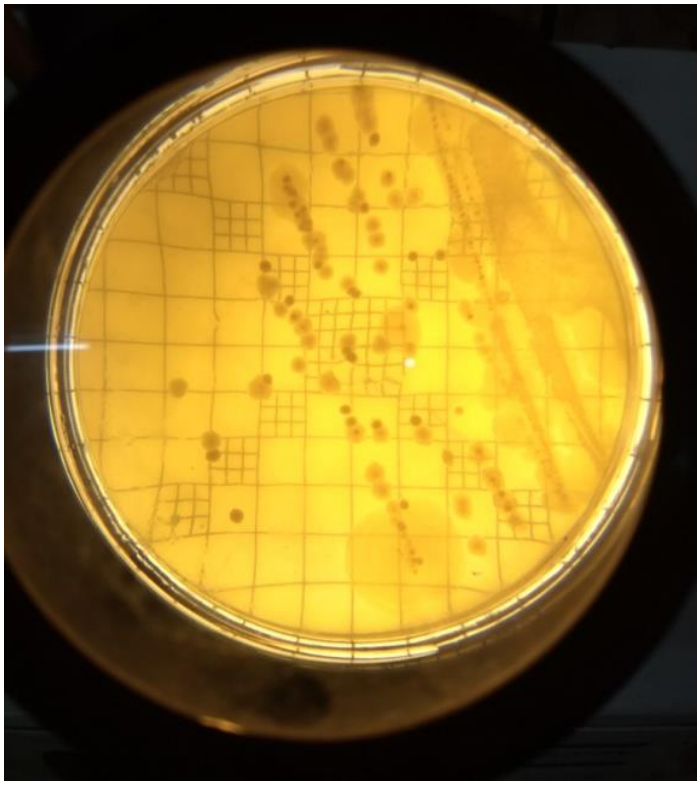
On analysing the result of antibiotic sensitivity test (AST) for different intestinal samples, it was found that the majority of eco-friendly microflora were sensitive to Gentamicin (82%), Vancomycin (73%) followed by Tetracycline (67%), Tobramycin (64%), Kanamycin (53%) Nalidixic acid (41%), Ampicillin (34%), Azithromycin (27%) and Metronidazole (21%) in normal MHA agar plate.

Table 4.7 Antibiotic sensitivity profile of various eco-friendly microbes isolated from different intestinal samples:

Serial no.	Name of Antibiotic Discs (mg)	Intestinal samples (n=57) showing sensitivity in MHA agar plate	Sensitivity (%)
1	Vancomycin (30mg)	41	73
2	Gentamicin (30mg)	47	82
3	Tobramycin (10mg)	36	64
4	Tetracycline (30mg)	38	67
5	Azithromycin (30mg)	15	27
6	Ampicillin (2mg)	19	34
7	Kanamycin (30mg)	30	53
8	Nalidixic Acid (30mg)	23	41
9	Metronidazole (5mg)	12	21

Fig . 4.10 shows antibiotic sensitivity profile of various eco-friendly microbes isolated from different intestinal samples





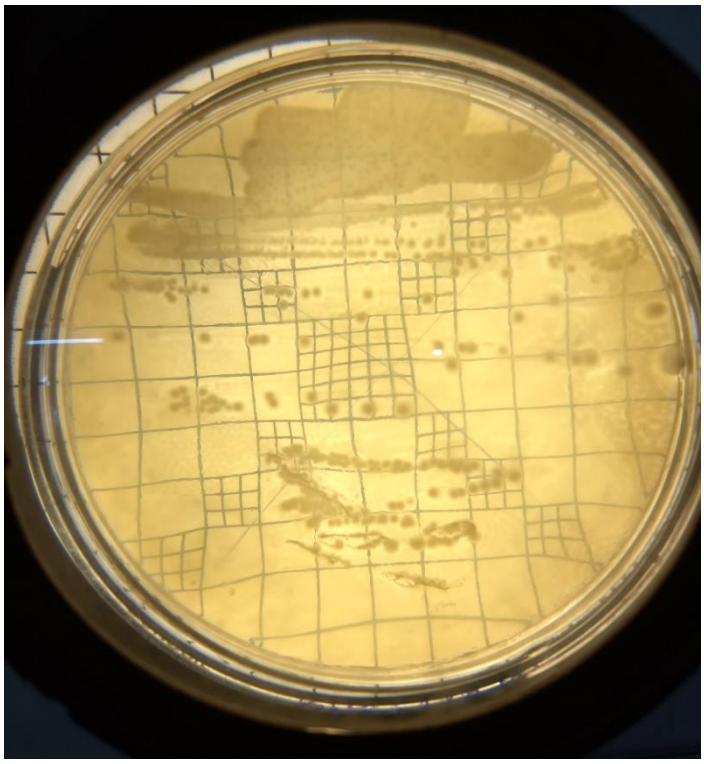


Fig. 4.11 and 4.12 : Appearance of bacterial colonies on MHA plate

DISCUSSION

In the present study, "Characterisation of eco-friendly micro-organisms in the gut microflora of chicks", a total no. of four hundred twenty five (n=425) samples consisting of intestinal and faecal samples were collected from different established and non established poultry farms present in and around Bhubaneswar, Odisha which includes OUAT Instructional Livestock farm and Central Poultry Developmental Organisation (CPDO). The above samples were collected from day old chicks upto 4 weeks in weekly interval from four different breeds like Kalinga Brown, Banaraja, Colour Cross and Hubbard. The samples were processed by routine microbiological procedures as per the method of Cruickshank et al. for bacterial isolation and identification.

As per various researchers, a diverse microbial community in the housing environment is carried over from one flock to the next and thus can serve as an important inoculum for the chick gastrointestinal microbiome in most of the commercial poultry operations (Liljebjelke et al., 2003). Newly hatched chicks coming from hatcheries have no contact with adult birds, and thus environmental microbial communities, of which the litter is likely to be the most important, function as important inocula that can shape the development of the gastrointestinal microbiome and potentially carry through the life of a flock. Through the life span of a commercial broiler (typically 42 days), significant changes in the taxonomic composition of the gastrointestinal microbiome have been observed (Lu et al., 2003).

From a total 81 no. of jejunum samples 48 no. of samples showed predominance of *Lactobacillus* species indicating 59.3% prevalence which was followed by 13.5% prevalence of *Clostridium* species. Similarly in 53 samples out of total 75 no. of ileal samples *Lactobacillus* species was abundant showing prevalence of 70.8 % while *Clostridium* species was found to be 10.7% prevalent. But in caecum and colon *Clostridium* species was found to be abundant showing prevalence of 64.7% and 57.5% respectively. These findings support the conclusion drawn by many researchers like Salanitro et al.,(1978); Jangrang et al.,(2003).

It was revealed from the current study that 16 no. of samples were collected from day old birds out of which 6 samples showed prevalence of *Clostridium* species (37.5%) where *Lactobacillus* species was found to be 25% prevalent. *Bacteroides* species showed 12.5% prevalence while *Bifidobacterium*, *Enterococcus*,and *Corynebacterium* species each showed 6.25% prevalence.

Similarly, 16 no. of samples were collected from 1 week age group of birds out of which 7 samples showed prevalence of *Clostridium* species (43.75%) where *Lactobacillus* species was found to be 18.75% prevalent. *Corynebacterium* species showed 12.5% prevalence while *Bifidobacterium* species, *Enterococcus* species and *Bacteroides* species each showed 6.25% prevalence.

19 samples were collected from 2 week age group of birds out of which 6 samples showed prevalence of *Clostridium* species (31.6%) where *Lactobacillus* species was found to be 15.8% prevalent. *Bifidobacterium* species showed 21.1% prevalence while *Enterococcus* species and *Bacteroides* species each showed 10.5% prevalence. *Corynebacterium* showed minimum prevalence of 5.25%.

17 no. of samples were collected from 3 week age group of birds out of which 4 samples showed prevalence of *Clostridium* species (23.6%) where *Lactobacillus* species was found to be 5.9% prevalent. *Corynebacterium* species showed 17.6% prevalence while *Enterococcus* and *Bifidobacterium* species each showed 11.8% prevalence. *Bacteroides* showed 23.6% prevalence.

17 no. of samples were collected from 4 week age group of birds out of which 6 samples showed prevalence of *Clostridium* species (35.3%) where *Lactobacillus* species was found to be 5.9% prevalent. *Corynebacterium* species showed 11.8% prevalence while *Bacteroides* and *Bifidobacterium* species each showed 17.6% prevalence. *Enterococcus* species showed 5.9% prevalence. This result is in accordance with the findings stated by Lorenzoni et al., (2011) and Zhou et al.,(2007).

In the study the biochemical characteristics of various eco-friendly bacteria were determined. *Lactobacillus* (Gram+ve) fermented glucose, fructose, lactose, maltose, arabinose and didn't ferment mannose. It produced gas on carbohydrate fermentation and didn't produce H_2S. Besides that it showed negative catalase and oxidase activity. Similary *Bifidobacterium*, a gram+ve bacteria fermented glucose,

fructose, lactose, maltose and mannose while it didn't ferment arabinose. It produced neither gas nor H_2S. It also showed negative catalase and oxidase activity. *Corynebacterium*, a gram+ve bacteria fermented glucose, fructose, lactose and maltose. It produced gas on glucose fermentation but didn't produce H_2S. It showed positive catalase activity and negative oxidase activity. *Clostridium*, the predominant bacteria of large intestine is a gram+ve one. It fermented glucose, fructose and mannose while it couldnot ferment lactose, maltose and arabinose. It produced both gas and H_2S. It showed neither catalase activity nor oxidase activity. *Enterococcus* , a gram+ve bacteria fermented glucose, fructose, lactose, maltose and mannose except arabinose. It didnot produce H_2S. Besides that it showed negative catalase as well as negative oxidase activity. *Bacteroides*, a gram-ve bacteria fermented glucose, lactose, maltose and didn't ferment fructose, mannose and arabinose. It didn't produce gas on carbohydrate fermentation while it could produce H_2S. It also showed both catalase and oxidase activity. Hereby it was concluded that all the eco-friendly bacteria isolated from the chicken intestine are glucose fermenters. The result was in agreement with the findings of Felten et al., (1999), Hadadji et al.,(2005).

The microbial load of various parts of the intestine such as jejunum, ileum, caecum and colon of four different chick breeds at various stages of growth were enumerated in the present study.

In jejunum of day old Kalinga brown chicks the microbial load was found to be 1.5×10^9cfu/ml. while in ileum the load was 1×10^9cfu/ml. In caecum the microbial load was 1.7×10^{10} cfu/ml. and in colon the load was 1.2×10^9 cfu/ml.. Similarly in 1st week the microbial load in jejunum, ileum, caecum and colon were 1.7×10^9, 1.5×10^9 ,2.1×10^{10}, 2.3×10^9 cfu/ml. respectively. In 2nd week the load estimated in jejunum, ileum, caecum and colon were found to be 2.3×10^9, 1.9×10^9, 2.4×10^{10}, 1.9×10^9 cfu/ml. respectively. In 3rd week the microbial load in jejunum, ileum, caecum and colon were 2.7×10^9, 2.8×10^9, 2.9×10^{10}, 2.9×10^9 cfu/ml. respectively. In 4th week the microbial load in jejunum, ileum, caecum and colon were 3.3×10^9, 3.7×10^9, 4.1×10^{10}, 3.2×10^9cfu/ml. respectively.

In the similar fashion the microbial load in different intestinal parts of Colour Cross breed at subsequent stages of growth was estimated. In day old Colour Cross chick the microbial load in jejunum, ileum, caecum and colon was found to be 1.3×10^9,

1.2×10^9, 1.4×10^{10}, 1×10^9 cfu/ml. respectively. In 1st week of age the microbial load in jejunum, ileum, caecum and colon was 1.8×10^9, 1.7×10^9, 1.8×10^{10}, 1.6×10^9 cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was 2.1×10^9, 2.2×10^9, 2.5×10^{10}, 2.3×10^9 cfu/ml. respectively. In 3rd weeks of growth stage the microbial load in jejunum, ileum, caecum and colon was found to be 2.6×10^9, 2.5×10^9, 2.7×10^{10}, 2.8×10^9 cfu/ml. respectively. In 4thweek the microbial load in jejunum, ileum, caecum and colon was found to be 3.2×10^9, 2.8×10^9, 3.9×10^{10}, 3.5×10^9cfu/ml. respectively.

In day old chicks of Banaraja the microbial load in jejunum, ileum, caecum and colon was 1.1×10^9, 1×10^9, 1.4×10^{10}, 1.3×10^9cfu/ml. respectively. In 1st week of age the microbial load in jejunum, ileum, caecum and colon was 1.7×10^9, 1.3×10^9, 1.9×10^{10}, 1.6×10^9cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 1.5×10^9, 2.1×10^9, 2.2×10^{10}, 2.6×10^9cfu/ml. respectively. In 3rd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 2.2×10^9, 2.9×10^9, 3.1×10^{10}, 1.5×10^9cfu/ml. respectively. In 4th week of age microbial load in jejunum, ileum, caecum and colon was 2.5×10^9, 3.8×10^9, 4.2×10^{10}, 3.5×10^9cfu/ml. respectively.

Lastly the microbial load in different intestinal parts of Hubbard breed at subsequent stages of growth was enumerated. In day old chicks the microbial load in jejunum, ileum, caecum and colon was 1.2×10^9, 1.3×10^9, 1.5×10^{10}, 1.4×10^9cfu/ml. respectively. In 1st week the microbial load in jejunum, ileum, caecum and colon was 1.7×10^9, 1.8×10^9, 1.9×10^{10} 2.1×10^9cfu/ml. respectively. In 2nd week of age the microbial load in jejunum, ileum, caecum and colon was estimated to be 2.3×10^9, 2.7×10^9, 2.8×10^{10},2.5×10^9cfu/ml. respectively. In 3rd week of age the microbial load in jejunum, ileum, caecum and colon was found to be 2.5×10^9, 2.1×10^9, 3.2×10^{10}, 3.4×10^9cfu/ml. respectively. In 4th week of age the microbial load in jejunum, ileum, caecum and colon was 2.8×10^9, 3.1×10^9, 4.5×10^{10}, 3.6×10^9 cfu/ml. respectively.

By analysing the results it was concluded that the average microbial load in jejunum was found to be highest in Kalinga Brown breed. Similary when the average microbial load of ileal contents were analysed ,it was observed that in Banaraja breed the microbial load in ileum was found to be maximum. In the similar fashion the average microbial load in caecum and colon was maximum in Hubbard breed.

The study revealed that out of 23 no. of faecal samples of Kalinga Brown breed analysed in different age groups such as day old , 1st week , 2nd week, 3rd week and 4thweek,the average microbial load was found to be 3.4×10^5, 4.1×10^5, 5.4×10^5, 6.7×10^5, 7.9×10^5 cfu/ml. respectively. Out of 21 no. of faecal samples of Colour Cross breed analysed in different age groups such as day old , 1st week , 2nd week, 3rd week and 4thweek,the average microbial load was found to be 2.5×10^5, 3.4×10^5, 4.1×10^5, 5.3×10^5, 6.9×10^5 cfu/ml. respectively. Out of 17 no. of faecal samples of Banaraja breed analysed in different age groups such as day old , 1st week , 2nd week, 3rd week and 4thweek,the average microbial load was found to be 3.1×10^5, 4.3×10^5, 4.8×10^5, 5.7×10^5, 6.3×10^5 cfu/ml. respectively. Similarly, out of 24 no. of faecal samples of Hubbard breed analysed in different age groups such as day old , 1st week , 2nd week, 3rd week and 4thweek,the average microbial load was found to be 2.6×10^5, 3.9×10^5, 5.8×10^5, 6.4×10^5, 8.5×10^5 cfu/ml. respectively. These results are in accordance with the reports of many researchers (Trawinska et al., 2016 ; Kostadinova et al., 2014).

By observing the average microbial load in faeces obtained at various stages of growth in different breeds it was concluded that at day old age maximum microbial load was found in faeces of Kalinga Brown breed. At 1stweek of age the average microbial load was maximum in faeces of Banaraja breed. At 2nd week of age the average microbial load was found to be highest in faeces of Hubbard breed. At 3rd week of age the average microbial load was highest in faeces of Kalinga Brown breed. At 4th week of age the highest average microbial load was found in faeces of Hubbard breed.

In the present study, qualitative methods of biofilm estimation like congo red agar method and tube method were used for the evaluation of biofilm forming abilities of the eco-friendly bacterial isolates. Congo red agar method was used as an indicator for initial screening of the isolates. In this method, when bacterial isolates were screened, it was found that out of 20 isolates 50% showed negative, 20% show moderate and 30% showed positive for biofilm production. So in this study it was observed that this method detected more negative values for biofilm production, which is not in

accordance with the findings that the congo red method is sensitive (Freeman et al., 1989).

In tube method, it was found that out of 20 isolates, 30% showed strong adherence, 30% showed moderate adherence, 5% showed weak adherence and 35% showed no adherence. This result correlates to a greater extent with the findings of Dheepa et al., 2011 in which it was stated that tube method detected 26% isolate as strong adherent, 34% as moderate adherent, 5% as weak adherent, 6% as negative. On comparision of the tube method with the congo red method, it was found that tube method is better than congo red agar method (Ruzicka et al. 2004).

On comparison of the above 2 methods, it was found that the percentage of positive values for biofilm production in tube method and congo red method were 65% and 50% respectively. So in the present study, it is concluded that tube method is the best method of biofilm assay in comparison to other method.

On analysing the result of antibiotic sensitivity test (AST) for 57 intestinal samples of different breeds of chicks, it was found that the majority of eco-friendly microflora were sensitive to Gentamicin (82%), Vancomycin (73%) followed by Tetracycline(67%), Tobramycin (64%), Kanamycin (53%), Nalidixic acid (41%), Ampicillin (34%), Azithromycin(27%) and Metronidazole (21%) in normal MHA agar plate.

Intestinal microbes are known to greatly influence the cost, development, and effectiveness of mucosal and systemic immune responses in chicks (Macpherson and Harris 2004). This trade-off is well evidenced by the fact that newly hatched germ free chicks have depressed immune functions. They have decreased cytokine production, systemic immunoglobulin levels, intraepithelial lymphocyte counts, and relative amounts of gut-associated lymphoid tissue (GALT).

Probiotics can be the most effective supplementation during the initial development of the microbiota and following antibiotic therapy and thus can be interpreted in the context of the ecological phenomena of primary and secondary succession in which a community is established. Several studies have demonstrated that supplementing feed with probiotic containing *Lactobacillus* cultures can enhance body weight gain and

feed efficiency and reduce the mortality rate in chicks (Zulkifli et al., 2000;Kalavathy et al., 2003; Timmerman et al., 2006).

SUMMARY

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In the present study, a total no. of 425 samples consisting of both intestinal and faecal samples were collected from different breeds of chicks such as Kalinga Brown, Colour Cross, Banaraja and Hubbard in various ages of growth such as day 0,1st week, 2nd week,3rd week and 4th week. Then they were subjected to routine isolation and identification procedure as per the method of Cruickshank et al. which revealed various types of microbes present in the chicken intestine and faeces with their load.

Out of total 81 no. of jejunum samples 48 no of samples showed predominance of *Lactobacillus* species which indicated 59.3% prevalence and similarly in 75 no. of ileum samples prevalence of *Lactobacillus* was found to be 70.8%. But the observation of 79 caecal samples and 73 colonic samples showed the abundance of *Clostridium* species indicating the prevalence of 64.7% and 57.5% respectively.

The study also revealed that *Clostridium* species was found to be abundant in intestine of chicks across different ages of growth. But the prevalence of *Lactobacillus* species gradually decreased in subsequent stages of growth period.

The biochemical characteristics of various eco-friendly bacteria such as *Lactobacillus, Clostridium*, *Bifidobacterium*, *Bacteroides*, *Enterococcus* and *Corynebacterium* species were also studied and it was found that the isolated species were predominantly gram+ve except *Bacteroides*. All of them were found to be glucose fermenters. *Corynebacterium* and *Bacteroides* showed positive catalase activity while only *Bacteroides* showed positive oxidase activity.

By analysing the microbial load of various parts of the intestine such as jejunum, ileum, caecum and colon of four different chick breeds at various stages of growth it was concluded that the average microbial load in jejunum was found to be highest in Kalinga Brown breed. Similarly when the average microbial load of ileal contents were analysed ,it was observed that in Banaraja breed the microbial load in ileum was found to be maximum. In the similar fashion the average microbial load in caecum and colon was maximum in Hubbard breed.

By observing the average microbial load in faeces obtained at various stages of growth in different breeds it was concluded that at day old age maximum microbial load was found in faeces of Kalinga Brown breed. At 1st week of age the average microbial load was maximum in faeces of Banaraja breed. At 2nd week of age the average microbial load was found to be highest in faeces of Hubbard breed. At 3rd week of age the average microbial load was highest in faeces of Kalinga Brown breed. At 4th week of age the highest average microbial load was found in faeces of Hubbard breed.

In CRA method of biofilm detection out of 20 isolates of *Lactobacillus*, 10 (50%) showed negative, 4 (20%) moderate and 6 (30%) positive for biofilm formation. Similarly in tube method of biofilm detection out of 20 isolates of *Lactobacillus*, 7 (35%), 1 (5%), 6 (30%), 6 (30%) showed no, weak, moderate and strong adherence respectively.

On analysing the result of antibiotic sensitivity test (AST) for 57 intestinal samples of different breeds of chicks, it was found that the majority of eco-friendly microflora were sensitive to Gentamicin (82%), Vancomycin (73%) followed by Tetracycline(67%), Tobramycin (64%), Kanamycin (53%), Nalidixic acid (41%), Ampicillin (34%), Azithromycin(27%) and Metronidazole (21%) in normal MHA agar plate.

Analysis of our own data indicated that clear successional changes in the taxonomic composition of the gastrointestinal microbiome can be observed during the life cycle of commercial broilers significantly associated with time. These findings potentiated the importance of age related microbial formulation in the form of probiotics. Probiotics can be enormously beneficial during the initial development of the microbiota and following antibiotic therapy and thus can be interpreted in the context of the ecological phenomena of primary and secondary succession in which a community is established.

The withdrawal of IFAs (Infeed antibiotics) from poultry feed requires the industry to look for various alternatives to maintain or improve the health and performance of birds and probiotics can be most effective solution for this.

CONCLUSION

Intestinal bacteria play an important role in pathogenesis of intestinal diseases since they are believed to protect against colonisation of the intestine and to stimulate the immune response of chickens.

Prevalence study during the research work revealed that eco-friendly bacteria like *Lactobacillus*, *Clostridium*, *Bifidobacterium*, *Bacteroides*, *Enterococcus* and *Corynebacterium* species are found in gastrointestinal tract of chicken of different breeds like Kalinga Brown, Colour Cross, Banaraja and Hubbard. *Lactobacillus* species was found more in ileum region as compared to other regions of GI tract. Similarly *Clostridium* species was found more concentrated in caecum region as compared to other regions of GI tract.

During the study of work *Clostridium* species was found more abundantly in GI tract in all age groups of chicks.

On faecal sample examination the maximum bacterial load was found in Kalinga Brown breed at the age of day old & and 3rd week, in Banaraja at the age of 1st week and in Hubbard at the age of 2nd& 4th week.

With respect to biochemical study it was found that *Corynebacterium* and *Bacteroides* showed positive catalase activity while only *Bacteroides* showed positive oxidase activity.

Out of 20 samples of *Lactobacillus*, 10 samples were shown positive for biofilm formation in Congo Red Assay method where as 13 samples were positive in Tube method.

With regard to antibiotic sensitivity test (AST) of 57 intestinal samples of different breeds of chicks, it was found that the majority of eco-friendly microflora were sensitive to Gentamicin (82%), Vancomycin (73%) followed by Tetracycline(67%), Tobramycin (64%), Kanamycin (53%), Nalidixic acid (41%), Ampicillin (34%), Azithromycin(27%) and Metronidazole (21%) in normal MHA agar plate.

Multidrug resistance in response to rampant use of antibiotics, has become an emerging problem in poultry industry which needs immediate attention . The present

study suggests for introduction of suitable microbial formulation in the form of probiotics at various stages of growth to stimulate the innate immunity of birds and increase growth performance.

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