METAGENOMIC ANALYSIS OF GUT MICROFLORA OF GUINEA FOWL FOR IDENTIFICATION AND EVALUATION OF PROBIOTIC *Lactobacillus* sp.

Thesis

Submitted to the
DEEMED UNIVERSITY
Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Poultry Science)

July, 2014

Dedicated to you Vinu…

March 3rd /2013, one of the brightest stars in my life faded away into the darkness. Today I am reflecting on my memories of him, and what he left behind.

केन्द्रीय पक्षी अनुसंधान संस्थान
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Dated: 10/7/, **2014**

Certificate

Certified that the research work embodied in this thesis entitled **"Metagenomic analysis of gut microflora of guinea fowl for identification and evaluation of probiotic *Lactobacillus* sp."** *submitted by* Dr. Vineetha P.G., Roll No. 5198, *for the award of* Master of Veterinary Science *degree in* Poultry Science *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

It is further certified that Dr. Vineetha P.G., Roll No. 5198, *has worked for more than 21 months in this Institute and has put in more than 150 days attendance under me from the date of registration for the degree of Master of Veterinary Science of the Deemed University, as required under the relevant ordinance.*

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Certificate

We the undersigned members of Advisory Committee of **Dr. Vineetha P.G.,** *Roll No. 5198 a candidate for the degree of* **Master of Veterinary Science** *with the major discipline* **Poultry Science**, *agreed that the thesis entitled* **"Metagenomic analysis of gut microflora of guinea fowl for identification and evalaution of probiotic *Lactobacillus* sp."** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

24/7/14

Signature

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Date: 24/7/14

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Place : IVRI, Izatnagar

(Vineetha P. G.)

ABBREVIATIONS

°C	Degree celsius
Ad lib	Ad libitum
AGE	Agar gel electrophoresis
BLAST	Basic Local Alignment Search Tool
BMD	Bacitracin Methylene Disalicylate
BW	Body weight
CARI	Central Avian Research Institute
CD	Crypt depth
CFS	Cell free supernatant
CFU	Colony forming unit
CP	Crude Protein
DCP	Di Calcium Phosphate
DNA	Deoxy ribonucleic acid
dNTP	2'-deoxy-nucleoside-5'-triphosphate
DORB	Deoiled Rice Bran
dpi	Day Post immunization
DW	Distilled water
EDTA	Ethylene Diamine Tetra Acetic Acid
ECM	Extracellular matrix molecules
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
GIT	Gastro Intestinal Tract
HI	Heamaglutination Inhibition
IBDV	Infectious bursal disease vaccine
IEL	Intraepithelial lymphocytes
ISR	Intergenic spacer region
LAB	Lactic acid bacteria
LGFCM	*Lacobacillus* Guinea Fowl Caecum
LGFCP	*Lacobacillus* Guinea Fowl Crop
LGFI	*Lacobacillus* Guinea Fowl Ileum
LGFP	*Lacobacillus* Guinea Fowl Proventriculus
L.I.*L.p*	Lab isolated *Lactobacillus plantarum*
LPA	Lymphocyte Proliferation Assay
LSU	Large subunit rRNA Database
ME	Metabolizable Energy

MgCl2	Magnesium Chloride
MG-RAST	Metagenome Rapid Annotations using Subsystem Technology
MOS	Mannan Oligosaccharide
MRS	De mann Ragosa Sharpe
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NSS	Normal Saline Solution
OD	Optical density
PBS	Phosphate Buffer Saline
PCI	Phenol-chloroform-isopropyl alcohol
PCR	Polymerase Chain Reaction
PHA-P	Phyto-haemagglutinuin P
RBC	Red Blood Cell
RD	Ranikhet Disease
RDP	Ribosomal Database Project
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SE	Salmonella Enteritidis
SPSS	Statistical Product & Service Solution
SSU	Small subunit rRNA Database
ST	Salmonella Typhimurium
TBE	Tris boric acid EDTA
TRIS	Tri hydroxy methyl amine methane.
UV	Ultra violet
VFA	Volatile fatty acid
VH	Villous height
W/V	Weight by volume
WHO	World Health Organization

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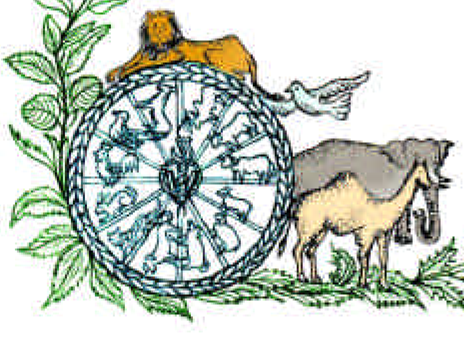


1

INTRODUCTION

The digestive tract of poultry contains a large numbers of microorganisms with a high diversity. The majority of these microbes are uncharacterized and represent an enormous unexplored reservoir of genetic and metabolic diversity. Traditionally, characterization of these microbial communities has relied on the ability to clonally culture each microorganism. But in recent past with significant improvements in nucleotide sequencing technologies (metagenomics) to economically obtain billions of bases, the investigation of microorganisms as a community regardless of their ability to be cultured has become reality. Metagenomics has been defined as function-based or sequence-based cultivation-independent analysis of the collective microbial genomes present in a given habitat (Riesenfeld *et al.*, 2004). This rapidly growing research area provided new insights into microbial life and access to novel biomolecules (Banik and Brady, 2008).

Using the metagenomic approach for analysis of guinea fowl intestinal microbes, through next generation sequencing (NGS) methods, we may enhance the understanding of communities of gut microorganism microbiome and thus about the health and diseases.

In the modern intensive poultry production, newly hatched chicks have little chance to contact with their mother, thereby normal microflora is slow to colonize in the intestine. This situation makes chicks likely to be affected by a small number of pathogenic bacteria due to sterile condition of intestine, then subsequently causing disease (Fuller, 1989). The disturbances in the balance of gut micro flora will obviously result in proliferation of pathogens. This scenario will in turn reduce the production performances of the animals or birds thereby causing financial loss to livestock and poultry producers (Pal and Chander,1999; Sauterand Blum, 2003). Extensive use of antibiotics and chemicals has resulted in environmental and human health concerns particularly with regard to the emergence of drug resistant pathogens and residual drugs in the food chain. Therefore, alternatives to the use of growth promoting antibiotics must be found to promote growth or production at or near the genetic potential of the modern day broiler, layers, guinea fowl, domesticated Japanese quail and turkey. In this context, the concept of probiotics has been evolved, which could be used in feed in order to maintain a balanced micro flora to enhance the health and productivity.

Lilly and Stillwell coined the term ‘Probiotics’ in 1965, which is derived from a Greek word ‘biotikos’ meaning ‘for life’. Probiotics are live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (FAO-WHO, 2001). According to Fuller, 1989, probiotic is a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance.

In recent year’s use of probiotics, prebiotics and synbiotics that enrich certain bacterial population in the digestive system are considered as alternatives to antibiotic growth promoters in poultry nutrition. (Patterson and Burkholder,2003). Probiotics, microbial cell preparations that are mono or mixed cultures of live protective microorganisms beneficially alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Helland *et al.*,2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients (Parvez *et al.,* 2006).

Similarly, prebiotics, which are non-digestible feed supplements, are selectively fermented by beneficial micro flora and are utilized by them to exclude the pathogenic microbes. The term ‘prebiotics’was introduced by Gibson and Roberfroid in 1995 defined them as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth of one or a limited number of bacteria in the colon.” These non-digestible substrates comprises of oligosaccharides considered as ‘food for beneficial microbes’ which on fermentation inhibit pathogens while simultaneously stimulating the absorption of several minerals in intestine.

Domesticated poultry raised under commercial conditions are vulnerable to a number of pathogens. The World Health Organization (WHO) has now urged egg and meat producers to use environment friendly alternative methods to control diseases

(Lowenthal *et al.*,1999). Hence every efforts were made to improve the gut efficiency of broiler and Guinea fowl through natural microflora and fauna for better performance.

In present scenario food scarcity is becoming a major issue, where supply is not able to meet the higher demand, we have to opt for diversification of poultry species such as guinea fowl, turkey, and domesticated Japanese quail etc. Guinea fowls are interesting gallinaceous birds being farmed for centuries to meet poultry production requirements of diversified eco-agriculture background. Guinea fowl production for meat is a potentially advantageous enterprise in many parts of the world (Nahashon *et al.,* 2006). Guinea fowl meat, as alternative meat to chicken, has already proven to be a profitable activity in the United States, Canada, and also in European markets such as France and Italy (Nahashon *et al.*, 2005; Tufarelli *et al.*, 2007).

However, there still remains a challenge in profitability emanating from increasing cost of production, which is primarily due to high FCR.With above mentioned beneficial effects of probiotics this problems can be addressed.

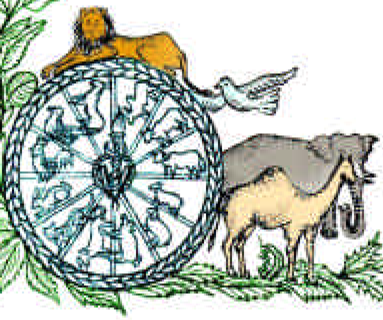
Since the literature on species specific *Lactobacillus* characterization and its effect as probiotic on performance in guinea fowl is insufficient, efforts were made to isolate, characterize and evaluate *Lactobacillus* spp from different segments of gastrointestinal tract of guinea fowl to improve the gut efficiency through natural microflora for better growth and immunity development.

In view of above, the present investigation was undertaken with the following objectives :

1. **To analyse the gut microflora from crop and caecum of adult guinea fowl birds using Next Generation Sequencing(NGS) and bio-informatics tools.**
2. **To identify and characterize *Lactobacillus* isolates from the GI tract microbiome of guinea fowl and screening of *Lactobacillus* isolates for their probiotic activity through various in-vitro assays.**
3. **To evaluate the probiotic properties of selected *Lactobacillus* species *in vivo ie, through* feeding trials in guinea fowl and broiler.**

✍ ✍ ✍

Review of Literature



2

REVIEW OF LITERATURE

An unabridged survey of literature reports on the subject is a pre-requisite to understand the importance of research work and for its further planning that may provide answer to many unsolved problems. In view of its importance the subject has been reviewed periodically. Accordingly an exercise has been made to review the pertinent reports published on the main subjects those with direct or indirect relevance to use of probiotics for poultry. Such information has been set out appropriately here in.

2.1. Guinea fowl

The term “guinea” fowl is the common name of the seven species of gallinaceous birds of the family Numididae, which is indigenous to Africa. The strains are descended from the helmeted guinea fowl, *Numida meleagris*. Of the three domestic varieties (the pearl, the white and the lavender), the purplish coloured pearl is the most common. In many parts of the world, guinea fowls are raised mainly for their gamey flesh and eggs. They were introduced into the Indian sub-continent during the slavery era of mediaeval centuries. Guinea fowl is a promising genetic resource for evolving a low input-grain saving poultry alternative for production in the developing world (Moreki,2009).Intimate understanding of the basics of domestication; disease control, nutrition and genetics is essential for commercial exploitation of this avian species.

2.2. CARIBRO –Dhanraja

CARIBRO–Dhanraja is a commercial cross developed by crossing coloured synthetic male and female lines at Central Avian Research Institute, Izatnagar.Average weight at at day old is found to be around 46g and at 6 weeks of age they attain a weight of 1600 to 1650g .Dressing percentage is as high as 73%, livability percentage is around 97-98% The Feed conversion ratio of CARIBRO_Dhanraja birds was found to be 1.90 to 2.10 at 6 weeks of age.

2.3. Metagenomics

The composition of the gastro intestinal tract microbiome reflects co-evolution among the inhabiting microbes, genetic, immune, and metabolic interactions with the host, and environmental influences (Yeoman *et al.,* 2011). Microbes are found across the entire length of the GIT, where they show spatial variation in community composition biogeographically as well as between luminal and mucosa-associated populations (Gong *et al.,* 2002). The intestinal microbiome in poultry GIT functions as an interface between the host and the feed ingested and has important roles in nutrition and health of the host. This intestinal microbiome consisting of bacteria, archaea, fungi, virus and few protozoa, with bacteria being the most abundant domain and greatly affecting the performance of the host. Modulation of the intestinal microbiome of poultry through dietary and managerial interventions has been used to improve poultry growth and health. However, the diversity and complexity of the intestinal microbiome hinders understanding of the mechanism of these interventions and achieving the intended modulations.The majority of poultry intestinal microbiome are uncultivable limits the knowledge that can be learned about this microbiome.

Metagenomics is a comprehensive approach to study both the structure (composition) and function of gut microbiota by giving sequence information from the collective genomes of the microbiota in a single experiment. The term 'metagenomics' was first coined by Jo Handelsman in l998. Metagenomics strives to determine the abundance and identity of microbes in a sample. The application of this technology resulted in the generation of large datasets derived from various places including gastrointestinal tract.

The Sanger methodology dominated the industry for over two decades leading to significant accomplishments and technological improvements in DNA sequencing. Next generation high throughput sequencing technologies were thus developed to overcome the limitations of this 'first generation' technology that include higher speed, less labor, and lowered cost. There are two approaches in sequencing: amplicon sequencing and shotgun sequencing. In amplicon sequencing, an informative marker such as the 16S rRNA gene is amplified by polymerase chain reaction (PCR) and sequenced. Shotgun sequencing refers to DNA that has been extracted and randomly sheared into smaller fragments before sequencing. Various platforms developed include sequencing-by-synthesis 454 Life Sciences, Illumina (Solexa) sequencing, SOLiD sequencing (among others) and the Ion Torrent semiconductor sequencing technologies that use different detection principles. Of the NGS technologies, both the 454/Roche and the Illumina/Solexa systems have now been extensively applied to metagenomic samples (Mardis *et al*.,2008).Genes of interest continue to include ribosomal RNA genes, genes that have been favoured for phylogenetic analysis since the early days of molecular microbiology, and which continue to be used as phylogenetic 'anchors' for further analysis of diversity and function (Tringe & Rubin.,2005). The 16S rRNA gene occurs in all living organisms, represents more than 80% of total bacterial RNA. The 16S rRNA gene includes interspersed conserved and variable regions, which makes it well suited for PCR amplification and sequencing. In this process, probes are designed to hybridize to the conserved regions, allowing for amplification and sequencing of the variable regions.

Bioinformatics is the application of computer technology to the management of biological information. MEGAN is a computer program that allows laptop analysis of large metagenomic data sets. In a preprocessing step, the set of DNA sequences is compared against databases of known sequences using BLAST (Altschul *et al.,* 1990). MEGAN is then used to compute and explore the taxonomical content of the data set, employing the NCBI taxonomy to summarize and order the results (Daniel *et al*., 2007).

2.4. *Lactobacillus* as probiotic

The microbial population in the gastro-intestinal (GI) tract is very complex and consists of different groups of microbes and the GI system is the place where complex interactions occur between feed, microbes and host cells (Dhama *et al.,* 2008). The health and nutritional status of poultry is largely interlinked with the gastrointestinal (GI) microflora, which directly or indirectly affects gut morphology, nutrition, the pathogenesis of intestinal disease, and immune responses .Bacteria are more commonly reported as probiotic than fungi. Two genera of bacteria are mostly reported including lactic acid bacteria of the genus *Lactobacllus* (Dalloul *et al.,* 2003, 2005; Yegani and Korver., 2008; Higgins *et al.,* 2008; Haghighi *et al.,* 2008; Taheri *et al.,* 2009; Lee *et al.,* 2010) and Bifodobacteria (Patterson and Burkholder., 2003). Microbial probiotics are commonly administered to animals orally either through the feed or drinking water. Among the potential probiotics, lactic acid bacteria (LAB) is reported to have important effects on animal performance (Chou and Weimer., 1999). They are generally regarded as safe (GRAS), and they display antagonistic activity against pathogenic bacteria. Lactobacilli represent an important component of the gut microflora in chickens. In birds, predominant species of *Lactobacillus* (*L. reuteri, L. salivarius,* and *L. animalis*) functions mainly as beneficial micro flora that are capable of competitively excluding pathogenic microbes from the gastro-intestinal (GI) tract.

The use of probiotics in poultry was pioneered by Tortuero *et al.,* (1973) who reported an increase in growth rate in chicks given a *Lactobacillus acidophilus* culture in drinking water for 11 days from hatching. Recent researches have suggested that the chicken gastrointestinal micro flora may contain more than 650 species of which more than half are from previously unknown bacterial genera (Apajalahti *et al.,* 2004). Some of the bacterial species isolated from the crop of chickens include *Escherichia coli, Enterococci, Staphylococci, Lactobacilli, Campylobacter* spp. and *Salmonella* spp. (Frei *et al.,* 2001) however many of these are transient species and later the dominant species found in the crop are lactobacilli (Van der Wielen *et al.,* 2002). Many reports showed that lactic acid bacteria (LAB) could be used as probiotics for animals (Chen *et al.,* 2005). The beneficial effects of *Lactobacillus* cultures on the growth of chickens were also reported by several researchers (Kalbane *et al.*, 1992; Jin *et al.*, 1998).

The Lactobacillales is the largest order in the phylum Firmicutes and the class Bacilli. The family Lactobacillaceae, directly under the Lactobacillales order (Hammes & Hertel, 2003).The genus *Lactobacillus* is the largest among the lactic acid bacteria (LAB), comprises a large and diverse group of gram positive, nonspore forming, catalase negative rod bacteria, with low G+C content, able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Pelinescu *et al.*, 2009). They are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications (Fuller, 1989). Lactobacilli are generally aero-tolerant anaerobic, aciduric or acidophilic. Optimum growth temperature and pH are usually 30-40°C and 5.5-6.2, respectively. It is one of many friendly species of intestinal microflora considered as beneficial bacteria in its ability to aid in breakdown of proteins, carbohydrates and fats in food and help absorption of necessary elements and nutrients such as minerals, amino acids and vitamins by the host.

According to metabolism, *Lactobacillus* species can be divided into three groups:

1. **Obligately homofermentative (Group I)**

   It includes *L. acidophilus, L. delbrueckii, L. helveticus, L. salivarius.*

2. **Facultatively heterofermentative (Group II)**

   It includes *L. casei, L. curvatus, L.plantarum, L. sakei*

3. **Obligately heterofermentative (Group III)**

   It includes *L. brevis, L. buchneri, L. fermentum, L. reuteri.* (Bergey's manual of determinitive bacteriology, 1994).

Utilizing 16S rRNA gene sequencing, microbiota from different regions of the gastrointestinal (GI) tract of birds has been analyzed on which, *Lactobacillus aviarius* and *Lactobacillus salivarius* were found to be the predominant species among *lactobacilli* (Gong *et al.,* 2007).

2.4.1. Screening of *Lactobacillus* isolates towards probiotic use

Many probiotics have been introduced by screening the natural intestinal microflora of poultry species. Determination of an optimal species for probiotic usage is completely empirical (Ehrmann *et al.,* 2002) and some criteria must be considered for selection. The important criteria consist of functional characteristics such as adhesiveness of *lactobacilli* to the gut epithelial cells, the resistance to environmental conditions of the digestive tract, and production of preventive substances against the pathogenic bacteria. The last property is considered as important ecological factor that determine dominant bacteria in some ecosystems like the intestine (Busarcevic *et al.,* 2008). The selected bacterial strains must be genetically stable and exhibit an adequate growth rate both *in vivo* and *in vitro* conditions.

2.4.2. Strain characteristics and habitat specificities in intestinal tract

The microbial ecology of chicken GIT is quite unique. At maturity, the chicken GIT is quite diverse consisting mostly of bacteria and to a lesser extent protozoa and fungi (Gabriel *et al*., 2006). The diversity/composition of the microbial flora of chicken GIT depends on several factors including diet composition, age of the chicken, breed, geographic location and the specific section of the GIT such as small intestine, ileum, cecum (Apajalahti *et al*., 2004). Different strains of probiotic bacteria may exert different effects based on specific capabilities and enzymatic activities, even within one species (Bernet *et al*., 1993, Ouwehand *et al*., 1999). It has been variously reported that each region of the chicken intestine develop its own unique microflora (Yegani and Korver, 2008). Different microorganisms express habitat preferences that may differ in various host species (Freter, 1992). *Lactobacilli* are among the indigenous flora colonizing the chicken's crop, the stomach of mice and rats, and the lower ileum in man. Bacteria colonizing such high transit rate sites must adhere firmly to the mucosal epithelium (Savage, 1972; Fuller, 1973 and Beachey, 1980) and must adapt to the milieu of this adhesion site.

2.4.3. Morphological characterization of *Lactobacillus* isolates

Lactobacilli are gram positive, nonspore forming, long slender rods to short coccobacilli forms (Hammes & Hertel, 2003). The color of colonies may vary from creamy, white, creamish white and greyish white for different isolates. Shape of colonies varies from pin point, large, circular, smooth, compact and convex, rough and irregular with entire or undulate margin. Size of colony ranges from 0.8-2.3 mm (Aarti *et al.,* 2012).

2.4.4. Physiological characterization of *Lactobacillus* isolates

Aarti *et al.,* 2012 found that 37°C is the optimum temperature for all the isolates and few of studied could either survive or grow at 10°C/42°C or at both the temperatures away from the optimum. At 4% NaCl conce. some isolates showed growth while at 6.5% no isolate could grow in the study.

2.4.5. Biochemical characterization of *Lactobacillus* isolates

Lactobacilli are catalase negative organisms. Other biochemical tests which can be used for identification are esculin hydrolysis, citrate utilization, fermentation profile of isolates for various sugars like arabinose, cellobiose, fructose, galactose, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, trehalose, salicin, sorbitol, inulin,glycerol,adonitol arabitol,sucrose and xylose etc (Aarti *et al.,* 2012).

2.4.6. Molecular characterization of *Lactobacillus* isolates

Although 16S ribosomal RNA gene sequence comparisons are used to determine phylogenetic relationships among *Lactobacillus* species (Kullen *et al.,* 2000 and Sui *et al.*, 2002), often the discriminating power of the 16S/23S r-RNA gene intergenic spacer region (ISR) is required for the identification of *Lactobacillus* species and strains (Berthier and Erlich., 1998; Blaiotto *et al.,* 2002; Song *et al.,* 2000; Tannock *et al.,* 1999 and Tilsala-Timisjarvi and Alatossava, 1997).

2.4.7. Survival in the gastrointestinal tract

The acidic condition of crop, proventriculus and gizzard may adversely affect the survival of bacteria in bird's gastrointestinal tract.Thus it has been suggested that microbial cultures that is to be used as probiotic should also screened for their acid resistance (Conway *et al.*, 1987). *Lactobacillus* sp show a moderate tolerance to acid pH during 90 min incubation which is decreased after 2 h but individual strains vary considerably (Charteris *et al.*, 1998). Acid tolerance can be mediated by membrane ATPases as described for *L .acidophilus* (Lorca and Font de Valdez., 2001). Garriga et al. (1998) screened LAB with regard to their tolerance to pH 3, and they showed that pH 3 did not decrease the number of LAB. Also, Jin *et al.* (1998b) found that all isolated lactobacilli have moderate or good resistance to pH 3 and the survivability of lactobacilli decreases under high acidic conditions, especially when lower than pH 2. Bile that is secreted to the duodenum may affect survival of probiotic bacteria. Bile acids are amphipathic molecules with antimicrobial potential that act as a detergent and interfere withbiological membranes (Lebeer *et al.*, 2008). In LAB, bile resistance appeared to be mediated by bile salt hydrolysis (McAuliffe *et al.*, 2005; De Boever *et al*., 2000). Bile salt hydrolase enzyme (BSH) activity has been found in many species including Lactobacillus (Gilliland and Speck., 1997) which decreases the solubility, and thus weakening the detergent effect of bile salts. The bile salt hydrolase enzyme is able to deconjugate bile salt to amino acids and cholesterol which lead to the reduction of the toxicity of bile acids on bacteria (De Smet *et al.,* 1995).

Idoui *et al.,* (2009) reported that sixteen lactobacilli strains were isolated and identified from poultry crop and examined for their potentiality probiotic properties and results showed that the strain tested were resistant to pH3, resistant to a number of antibiotics and were also bile tolerant.

2.4.8. Secretion of enzymes

The probiotic strains stimulate host enzymes involved in the digestion of complex nutrients, or provide a probiotic source of these enzymes. Also, probiotics can synthesize vitamins and other essential nutrients not provided in sufficient quantities in the diet.

The microbes of probiotic in the intestinal tract of the inoculated birds secrets the amylolytic, cellulolytic, proteolytic and lipolytic enzymes (Jozefiak *et al.,* 2004; Lazaro *et al.,* 2003; Bedford, 2001), which provide maximum help to enhance the digestibility of starch, protein and fat components in the sequential way and liberated maximum energy. Such energy would not only improve the overall vital activities in the birds, but also improve the body weight (Nava *et al.,* 2005; Chiang and Hsieh.,1995). Omprakash *et al.,* (1996) also found the highest body weight (500 to 550g/chick) by incorporating probiotic at 15 or 20 ml/litre drinking water in broilers, at starter phase. As per the results of many researchers works (Jozefiak *et al*., 2004; Kabir *et al.,* 2004; Lazaro *et al.,* 2003; Shoeib and Madian, 2002; Sklan., 2002; Cross., 2002), they also predicted that the all beneficial species of *Lactobacillus* if added in the form of probiotic had the efficiency to produce energy nutrients digesting enzymes, which could be able to accelerate the catalytic activities of the endogenous enzymes and generate higher content of energy thereby improving body weight in broiler chicks during starter phase.

Taheri *et al.* (2009) investigated enzymatic activities of 332 lactic acid bacteria of broiler gut origin and no lipase activity was detected, but all of them showed almost similar proteolytic activities. However, they had different amylase and phytase activities. Supplementation of *Lactobacillus* cultures to chicken increased the amylolytic activity in the intestine supports the previous finding of author that *Lactobacillus* spp. are able to produce amylase extracellularly and intracellularly but extracellular protease and lipase of the *Lactobacillus* spp. were not detected both *in vitro* and *in vivo* test. (Jin *et al.,* 2000). The beneficial effects of expression of á-amylase, phytase, α-glucanase, xylanase, and cellulose enzymes in the *Lactobacillus* (Scheirlinck *et al.,* 1990; Liu *et al.,* 2005, 2007; Yu *et al.,* 2008) show the importance of enzymatic activities in bacterial strains of chicken probiotics.

2.4.9. Antagonistic test

Environment of GI tract is suitable for growth of pathogenic bacteria if pH of GI tract goes toward the basic (Payne *et al.,* 2007; Presser *et al.,* 1997). Any decrease of pH due to lactic acid inhibits the growth of pathogens. The production of antimicrobial agents could be easily demonstrated *in vitro* by the disc diffusion assay; they include fatty acids, organic acids, hydrogen peroxide, diacetyl, acetoin and the small heat-stable inhibitory peptides called 'bacteriocins'(Simova *et al.*, 2009; Soomro *et al.*,2002).

2.4.10.Cell surface properties mediating adhesion and coaggregation

Aggregation and cell surface hydrophobicity of the bacteria could be used instead of the examination of adhesion ability to the mucus (Taheri *et al.*, 2009), because there is a strong relationship among these characteristics especially between aggregation time and adhesion ability to the epithelium of the digestive tract (Garriga *et al.,* 1998). A correlation of the hydrophobic nature of the outermost surface of bacteria has previously been implicated in attachment of bacteria to epithelial and intestinal mucosal cells (Rosenberg *et al.*, 1983).Aggregation and cell surface hydrophobicity of the bacteria could be used for Mechanisms of adherence to an epithelial surface involve both receptor-specific binding and charge and hydrophobic interaction. LAB coaggregate with cells of the same strain or with cells from other species (Kolenbrander., 2000; Roos *et al*., 1999; Kmet *et al*., 1995). There are many studies which have confirmed a positive correlation between hydrophobicity and adhesion (Ehrmann *et al*., 2002; Handley *et al.,* 1987; Slomiany and Slomiany., 1984; Rosenberg *et al.,* 1983) and between aggregation and hydrophobicity (Rahman *et al.,* 2008; Bujnakova *et al.*, 2004). Therefore, it is reasonable to assume this mechanism may facilitate adhesion, e.g. to mucus. *Lactobacillus* can also express binding of extracellular matrix molecules (ECM), like collagens, fibronectin and vitronectin which may be shed from the epithelium to the mucus layer, and to mucus components (Lorca *et al*., 2002A; Sillanpaa *et al*., 1995; Aleljung *et al.*, 1994).

2.4.11.Tolerance to antibiotics

Antibiogram profile is also important, since many antibacterial chemicals are used as feed additives in poultry farms. As with any bacteria, antibiotic resistance exists among some lactic acid bacteria, including probiotic microorganisms (Salminen *et al*., 1998). Zhoua *et al*. (2005) reported that many strains of lactobacilli were resistant to Gram-negative spectrum antibiotics and aminoglycosides. Hummel *et al.* (2007) reported that lactobacilli seem to be intrinsically resistant to quinolones. Generally lactobacilli seem to be sensitive to penicillins (Danielsen and Wind, 2003). Lactobacilli are generally susceptible to antibiotics that inhibit the synthesis of protein, such as erythromycin and tetracycline (Essid *et al.*, 2009).Taheri *et al.* (2009) reported the antibiotic resistance property of *Lactobacillus johnsonii* LT171 to nalidixic acid and neomycine.

2.4.12.Fermentation of basal feed with *Lactobacillus* culture

Feed is probably the most important entity in the poultry industry that can expose the birds to a wide variety of factors through the gastrointestinal (GI) tract.The nutritional properties of fermented feed depend on the fermented starter (bacteria culture used to start fermentation), substrates, and fermentation conditions (temperature and incubation time) (Awati *et al.*, 2006). Savvidou *et al.* (2009) investigated the effect of fermented liquid feed with high numbers of *lactobacilli* (>10^9 cfu /g) and fermented liquid feed was prepared by mixing commercial unmedicated pelleted feed with water at a ratio of 1 feed: 1.2 water, inoculating with 10^6 cfu of *L. salivarius* and incubating at 30°C for 24h.

2.4.13.Effect on growth performance and carcass traits

Baghel and Singh (2004) opined that the probiotics have been found to improve the production performance of poultry, establish an environment to increase the digestibility of feeds and were a potential alternative to antibiotics in poultry diet. Salarmoini *et al.* (2011) investigated the effects of *Lactobacillus acidophilus* containing 2×10^8 cfu and found that feed intake in chicks fed diet supplemented with commercial probiotic was significantly higher than *L. acidophilus* probiotic. Chimote *et al.* (2009) and Tortuero *et al.* (1973) reported an increase in growth rate in chicks given a *Lactobacillus acidophilus* culture. Ghavidel (2011) found that the *lactobacillus* supplements significantly increased body weight and decreased feed intake and the mortality rate in broilers but no effect of probiotic on the relative weights of edible internal organs, thigh, liver, abdominal fat and losses carcass percentage were found.

Kalavathy *et al.* (2005) investigated the effect of mixture of *Lactobacillus* cultures (LC) on fat and fatty acid composition in the liver, muscle and carcass of broiler chickens and reported that the fat contents of the liver, muscle and carcass were also significantly lower in the LC-fed broilers when compared to the control broilers. Meat from probiotic fed birds showed lower total viable count as compared to the meat obtained from control birds and mean values of giblets; hot dress weight, cold dress weight and dressing percentage were significantly higher for probiotic fed broilers (Mahajan *et al.*,1999)**.**

2.4.14.Effect of *Lactobacillus* on intestinal histomorphology

Effects of the probiotic on villus height may change depending on the species of microorganism or probiotic. For example, villus height in duodenum and ileum increased but did not changed in jejunum of broiler chicks supplemented with *Pediococcus acidlactici* as probiotic (Taheri *et al.*, 2010). On the other hand Gunal *et al.* (2006) reported that villus height of jejunum and ileum increased in broiler chicks applied multi-microbe probiotic product. The villus height, villi perimeter in the ileum, and mucosa height in the cecum were significantly greater for the 42 day-old broilers treated with the probiotic *L. reuteri Pg4* (Yu *et al.,* 2007).

Awad *et al.* (2010) have reported that villus height to crypt depth ratio increased in duodenum and ileum of chicks supplemented with *Lactobacillus* sp. The increase in the villus height and villus height:crypt depth ratio was associated with improvement of growth performance for both synbiotic and probiotic. Similarly, supplementation of multi-microbe probiotic product has been reported to increase villus height to crypt depth ratio in duodenum and ileum (Kim *et al.,* 2011). It has been indicated that, increased villus height to crypt depth ratio are directly correlated with an increased epithelial turnover (Fan *et al.,* 1997). Awad *et al.* (2006) observed that the histological alterations caused by deoxynivalenol (DON) were reduced by supplementing the DON containing diets with probiotic feed and led to comparable villus length as in the control group. Patra *et al.,* 2013 also reported an increase in villous height and cryptal depth of ileum of domesticated Japanese quails received lower dose of commercial probiotic.

2.4.15.Effect of *lactobacillus* on microbial ecology-competitive exclusion

The term competitive exclusion was actually coined by Lloyd *et al.* (1974), who first applied it to poultry. Nurmi and Rantala, (1973) introduced the method of “competitive exclusion” (CE) to increase the resistance of young chicks to Salmonella infection by inoculating them orally with intestinal content from adult birds.They demonstrated that oral inoculation of two day old chicks with a 1:10 dilution of normal intestinal contents from healthy adult birds one day prior to oral challenge with S. infantis resulted in 77% of birds free from infection.

Proposed mechanisms of pathogen inhibition by the probiotic microorganisms include competition for nutrients, production of antimicrobial compounds such as bacteriocins, organic acids, volatile fatty acids and hydrogen peroxides. As probably secondary effects, modifications of the structure and function of the intestinal epithelium were also described.

Experiments with gnotobiotic chicks have confirmed that lactobacilli exert a controlling effect on *E. coli* (Fuller, 1978).Probiotic supplementation of the intestinal microflora in poultry, especially with Lactobacillus species, showed beneficial effects on resistance to infectious agents such as Escherichia coli (Jin *et al.*, 1996), Salmonella sp. (Pascual *et al.*, 1999), Campylobacter sp. (Stern *et al.*, 2001) and more recently, *Eimeria acervulina* (Dalloul *et al.*, 2003). Salarmoini *et al.* (2011) conducted experiment to investigate the effects of *Lactobacillus acidophilus* on intestinal micro-flora of broiler chick and reported that the number of *Lactobacilli* in ileum and colon were higher in *L. acidophilus* treated birds than the control group and also the number of coliforms was lower but the effects were not statistically significant.

Mountzouris *et al.* (2007) investigated the efficacy of a multi bacterial species probiotic in broiler nutrition containing *Lactobacillus* strains. Concentrations of bacteria belonging to *Lactobacillus* were significantly higher in treatments i.e. probiotic feed in water (PFW) and PF (Probiotic in feed) as compared with the control and antibiotic treatments. In addition, treatments PFW and PF modulated the composition and to an extent, the activities of the caecal microflora resulting in a significant probiotic effect.

2.4.16.Effect of *Lactobacillus* on immune response

According to Brisbin *et al.* (2011), systemic antibody and cell-mediated immune responses can be modulated by oral treatment with *lactobacilli* but these bacteria may vary in their ability to modulate the immune response. Mohiti *et al.*, (2007) and Noverr & Huffnagle (2004) also reported that resident microbiota play a pivotal role in shaping the immune system repertoire. Dalloul *et al.* (2003) reported an immunoregulatory effect of lactobacillus based dietary probiotic on the local immune response as characterized by altered intestinal intraepithelial lymphocytes (IEL) subpopulations and increased the birds' resistance to *E. acervulina* as reflected by reduced oocyst shedding. Zulkifli *et al*. (2000) found that birds treated with a lactobacillus culture mounted a higher serum antibody response than the oxytetracycline-treated and the control birds. Birds fed with *lactobacillus* based dietary probiotic showed significantly higher antibody titers against IBDV (Naseem *et al.*, 2012).

Havenaar and Spanhaak (1994) reported that probiotics stimulate the cell mediated immunity of the chickens in two ways, flora from probiotic migrate throughout the gut wall and multiply to a limited extent or antigen released by the dead organisms are absorbed and this stimulate the immune system. (Muir, 1998) opined that the lactic acid producing bacteria could interact with M cells which activate Payer's patches lymphocyte to be liberated from the intestine and reach the circulation. Moreover, Kohler *et al.* (2003) recorded that the wall of lactic acid producing bacteria is mainly composed of peptidoglycans and polysaccharides stimulating macrophages to release IL2, IL1 which are mainly concerned with activation of lymphocytes.

2.5. Prebiotics

The concept of prebiotics essentially has the same aim as probiotics, which is to improve host health via modulation of the intestinal flora, although by a different mechanism. Synbiotics are defined as 'mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host' (Andersson *et al.,* 2001). Prebiotics have been shown to alter GI micro flora, alter the immune system, prevent colon cancer, reduce pathogen invasion including pathogens such as *Salmonella* Enteritidis and *E. coli* and reduce cholesterol and odor compounds (Cummings and Macfarlane 2002).

Macromolecules that are increasingly being investigated for their prebiotics activities includes mannan oligosaccharides, fructo-oligosacharides, inulin, transgalactooligosaccharide, gluco-glucooligosaccharide, xylo-oligosaccharide, glycololigosaccharide, lactulose, lactitol, maltooligosacharide, stachyose, raffinose, etc. (Patterson and Burkholder, 2003). Although mannan oligosaccharides (MOS) have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneûcial bacterial populations. Instead, they are thought to act by binding and removing pathogens from the intestinal tract and stimulation of the immune system (Spring *et al.,* 2000). MOS have proven effective at improving weight gain and feed conversion efficiencies while also protecting against infection through pathogen binding (Eseceli *et al.,* 2010). The Bio-Mos supplement improved body weight and feed efficiency comparable to antibiotics with significantly lower mortality in broiler chicken and turkey (Hooge, 2004).

Young birds fed on MOS diets or Zinc bacitracin diet had smaller (relative weight) liver than those birds fed on the negative control diet. The relative weight of the other visceral organs,including the small intestine, were not affected by MOS treatment (Yang *et al.,* 2007).

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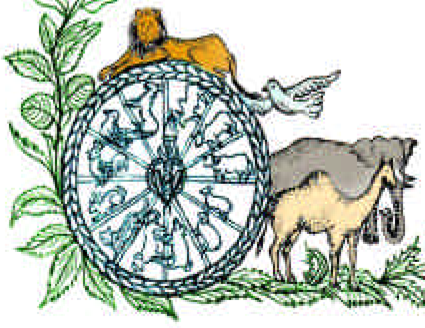
Materials and Methods



3

MATERIALS AND METHODS

In order to achieve the objectives, the plan of work was conducted at Guinea fowl unit, Experimental broiler farm and Avian Biotechnology Laboratory of Avian Genetics and Breeding Division, CARI, Izatnagar (UP). The test supplements employed and evaluated in this study were procured from local market and *L.acidophilus* obtained from NCDC (National Collection of Dairy Cultures), Karnal and isolated *lactobacillus* from GIT of adult guinea fowl of pearl variety. The research study has been done to incorporate the aspects in accordance with the guideline of “Institutional Animal Ethics Committee” (IAEC).

3.1. Germplasm

- Adult Guinea fowl– Pearl variety maintained at Guinea fowl unit,CARI, Izatnagar
- Broiler chicken– CARIBRO Dhanraja maintained at Broiler farm, CARI, Izatnagar

3.2. Chemicals and reagents

3.2.1. NGS

- 2X KAPA Hi-Fi Hotstart Ready Mix (KAPA Biosystems)
- Agencourt AMPure XP SPRI beads (Beckman Coulter)
- Nuclease Free Water (Ambion)
- High Sensitivity Bioanalyzer Kit (Agilent)
- Qubit DNA HS kit (Invitrogen)

3.2.2. *Lactobacillus* isolation

Bacteriological (Himedia) and Molecular grade chemicals and reagents from standard manufacturer (Amraesco,Sigma-Aldrich,Genetix etc.) were used for the study.

3.3. Equipments

Following equipments were used during the experimental period.

- Centrifuge (M/s Sigma)
- Thermal cycler (M/s Bio-Rad)
- Biosafety cabinet (Esco)
- CO_2 incubator (Med spec)
- Water purification system (M/s Alga)
- Cooling micro-centrifuge (3500)
- Table – top micro refrigerated centrifuge, (Cubota Corporation, Tokyo, Japan)
- Micropipettes (Eppendorf AG, Germany)
- Nano drop- 1000 (Thermo Scientific, Singapore)
- Gel documentation system (Biometra, UK; Syngene, USA)
- Horizontal submarine electrophoresis apparatus (Scie-Plas Ltd., Warwickshire, England)
- Double beam UV-VIS spectrophotometer (ICI, India)

3.4. Glass and plastic wares

Glass wares i.e. beaker, test tubes, cylinders, conical flasks and round bottle flasks etc. (Borosil, India) were utilized. Plastic wares viz. microcentrifuge tubes (0.2 ml, 1.5 ml and 2 ml) and tips (20 µl, 200 µl and 1 ml) procured from Axygen (USA) and Falcon tubes (15 ml and 50 ml) purchased from Axygen (India), Nunclon cell culture plates (Sigma Aldrich) were used.

3.5. Metagenomics

3.5.1. Collection of samples for Next Generation Sequencing (NGS)

For metagenomic analysis, sample was collected from adult guinea fowl housed in deep litter reared under uniform management and feeding conditions. Gut samples from the crop of 5 birds was collected for DNA isolation and sequence analysis. The contents of crop was collected into a sterile 15 ml tube containing about 10 ml of sterile PBS and pooled for DNA isolation and NGS analysis.

Table 1.1-1.5. PCR conditions employed in NGS for amplification of variable regions of 16Sr RNA

Table 1.1. Round1 Outer PCR for V3

	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3 min.	
Denaturation	98	30 sec	20
Annealing	60	60 sec	
Extension	72	60 sec	
Final Extension	72	5 min.	
Hold	4		

Table 1.2. Round 1 nested PCR for V3

	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3 min.	
Denaturation	98	30 sec	20
Annealing	45	60 sec	
Extension	72	60 sec	
Final Extension	72	5 min.	
Hold	4		

Table 1.3. Round1 PCR for V4

	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3 min.	
Denaturation	98	30 sec	30
Annealing	62	60 sec	
Extension	72	60 sec	
Final Extension	72	5 min.	
Hold	4		

Table 1.4. Table 1.1. Round1 PCR for V4V6

	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3 min.	
Denaturation	98	30 sec	30
Annealing	53	60 sec	
Extension	72	60 sec	
Final Extension	72	5 min.	
Hold	4		

Table 1.5.Round 2 PCR for addition of indexed primers

	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3 min.	
Denaturation	98	30 sec	3
Annealing	53	60 sec	
Extension	72	60 sec	
Denaturation	98	30 secs	7
Annealing	60	30 secs	
Extension	72	30 secs	
Final Extension	72	2 mins	
Hold	4		

3.5.2. DNA isolation for NGS

Samples were centrifuged at 700X *g* for 1 min for removing debris, and the supernatant was collected and centrifuged at 12000 *g* for 5 m. DNA was isolated using Qiagen Dneasy Blood & Tissue Kit. Pellet was washed twice with PBS and stored at -20°C. The quality control analysis of individual DNA samples were performed using Nanodrop, qubit fluorometer and gel electrophoresis.

3.5.3. Library preparation and sequence analysis

Region of V3-V4 and V5-V6 of 16s rRNA from individual metagenomic DNA was amplified using specific primers via PCR. Primer sequences used are given below.

1. V3(F):ACTCCTACGGGAGGCAGCAG
2. V3(R):TTACCGCGGCTGCTGGCAC
3. V4(F):AYTGGGYDTAAAGNG
4. V4(R):TACNVGGGTATCTAATCC
5. V4-V6(F):GTGCCAGCMGCNGCGG
6. V4-V6(R): GGGTTNCGNTCGTTG

(For V3 regions, an additional outer PCR was performed to amplify the larger V3 region before proceeding with the nested PCR. Universal primer sequences used for the outer PCR :

- V3-outer-Fw: V3 outer_F (27F): AGAGTTTGATCCTGGCTCAG
- V3-outer-Rev: V3 outer_R (1492R): TACGGTTACCTTGTTACGACTT

Library preparation was performed at Genotypic Technology's Genomics facility. Amplicons were constructed with the starting amount of 10 ng and 30 cycles of PCR was performed to amplify the region of interest using specific primers. The amplified region was cleaned up using Agencourt Ampure XP SPRI beads (Beckman Coulter). The equi-molar pooled amplified product was used for next round of PCR. Index barcodes were added using modified primers which had adapter sequences. 10 cycles of PCR was performed and the product was cleaned up using Agencourt Ampure XP SPRI beads (Beckman Coulter). The PCR products were purified on 2.0% gel and quantified spectrophotometrically. Also the prepared library was quantified using Qubit flurometer and validated for by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent). PCR conditions are given in Table 1.1,1.2,1.3,1.4,1.5.

The DNA from prepared libraries was denatured and sequenced on the Illumina MiSeq using sequencing by synthesis method to read 300 bases paired end. DNA library fragments were diluted, denatured and hybridized to a lawn of oligonucleotides immobilized on the flow cell surface. Hybridized DNA template was amplified using immobilized oligonucleotides as primers. Each hybridized template through the process of isothermal bridge amplification resulted in the formation of clusters comprised of roughly 1000 clonal copies. Sequencing was performed by synthesis (SBS) technology using four fluorescently labeled nucleotides to sequence each cluster on the flow cell surface in parallel. During each sequencing cycle, a single labeled deoxynucleotide triphosphate (dNTP) was added and clusters were imaged. The fluorescent dye and blocker was cleaved off and the next complementary base was added to the nucleic acid chain and imaged. 300 such cycles were performed which corresponds to 300 bases sequenced. Individual bases were called directly from signal intensity measurements during each cycle. These cycles comprised Read 1 of the sequencing run. Once Read 1 was completed, a second set of cluster generation took place on the MiSeq. Once the clusters were generated, sequencing was performed again on the reverse strand (Read 2).

Once sequencing was completed, the raw data was extracted from the server using the proprietary Illumina pipeline software to obtain FASTQ files. The reads generated were analysed using different programs viz. Greengenes, LSU, RDP, SSU, MG-RAST, Krona etc.

3.6. Isolation of *Lactobacillus* by Cultural Methods

3.6.1. Collection of samples for *Lactobacillus* sp. isolation

Samples were collected from adult Guinea fowl housed in deep litter reared under uniform management and feeding conditions. Gut samples from 5 birds were collected separately from crop, proventriculus, ileum and caeca for *Lactobacillus* species isolation. The contents were collected into a sterile 15 ml tube containing about 10ml of sterile PBS and pooled as per different segments of GI tract. The sample contents in the tubes were homogenized by vortexing and centrifuged at 700x for 5 min for removing debris. Supernatant was collected from each tube and aliquots (1ml) from each sample was enriched in MRS broth (De mann, 1960) and incubated for 48 h at 37°C in anaerobic jars. After that aliquots (100 µl) from each sample previously enriched with 9ml of MRS broth was plated on MRS agar. Thirty-two colonies, eight colonies each from crop, proventriculus, ileum and caeca were randomly picked up based on colonial morphology *i.e.,* selection based on colour, texture, size & shape of colony. Then each isolate was inoculated to MRS broth and incubated at 37°C for 48 h; subcultured in MRS broth 2 times for purification of *Lactobacilli* isolates. Then a loopful of inoculum of each isolate was streaked on to MRS agar and incubated at 37°C for 48 h, single colony from each plate was picked up and subcultured in MRS broth twice.The purified cultures were stored as 50% glycerol stock mixture (250 µl of broth culture in 1.75 ml of 50% glycerol+50% MRS broth medium) at -80°C until further use (Garriga *et al.,* 1998). The isolates were subcultured at least 2 times before all of the assays. 1 ml from each isolate culture was used for CFU enumeration.

After isolation of *Lactobacillus*, characterization was carried out by morphological, biochemical and molecular methods.

3.6.2. Morphological characterization

Phenotypic characterization of *lactobacillus* isolates was carried out by Gram staining (Gram, 1884) which differentiates bacterial species into two large groups (Gram-positive and Gram-negative). Also the color, shape, size of colonies were observed and noted.

3.6.2.1. Gram staining of *Lactobacillus* isolates

Gram’s staining differentiates bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls and divides the eubacteria into two fundamental groups according to their stainability and is one of the basic foundations on which bacterial identification is built was devised by Danish bacteriologist Hans Christian Gram (1853–1938) in 1882. Gram staining consists of four stains like primary stain (Crystal violet), mordant (Gram’s Iodine), decolourizer (Ethyl alcohol) and counterstain (Safranin) (Gram, 1884).

3.6.2.1.1. Procedure

The smear on a glass slide was covered with few drops of one of the primary stain like crystal violet which renders all the bacteria uniformly violet after a minute of exposure to the staining solution followed by washing the slide in water. The smear was treated with few drops of Gram's Iodine and allowed to act for a minute which results in formation of a dye-iodine complex in the cytoplasm followed by again washing in water. Then the slide was decolorized in absolute ethyl alcohol and the process is fairly quick and should not exceed 30 seconds for thin smears. Decolorization is the most crucial part of Gram staining and errors can occur so was carefully done. After the smear was decolorized, it was washed in water without any delay. The smear was finally treated with few drops of counter stain safranin and the slide was washed in water. The excess water was removed using a blotting paper, dried in air and heat fixed before observing under microscope. Bacteria that hold on to primary dye-iodine complex and remain violet are called Gram positive. Those which get decolorized and subsequently take up counterstain (pink/red) are called Gram negative.

3.6.3. Physiological Characterization

3.6.3.1. Effect of NaCl concentrations on growth of isolates

The isolates were inoculated in MRS broth having different NaCl concentration (2.0%, 4.0% and 6.5%) and incubated at 37°C for 48 h. The culture tubes were observed for the presence or absence of growth and results were noted as positive or negative.

3.6.3.2. Growth of isolates at 15 °C and 45 °C

The isolates were tested for their ability to grow in MRS broth at 15°C for and 45°C by incubating for 48 h. For this, 10 ml of MRS broth tubes were inoculated with 1% of *lactobacilli* isolates cultures. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at 15°C and 45°C and results were noted as positive or negative.

3.6.4. Biochemical Characterization

Biochemical tests were carried out to check the catalase enzyme activity through catalase test and carbohydrate fermentation by lactobacillus isolates through various sugar fermentation tests.

3.6.4.1. Catalase test

Hydrogen peroxide is a by-product of respiration and is lethal. The enzyme catalase degrades the hydrogen peroxide in the cell before it can do any cell damage. It splits the hydrogen peroxide to free oxygen which appears as bubbles and water. Generally the test reaction is very fast and is particularly important for the gram positive bacteria (Cowan and Steel, 1965).

3.6.4.1.1. Procedure for Catalase test

The inoculum was picked from a plate culture and was placed on a slide. One drop of 3% hydrogen peroxide was added and looked for immediate bubbling. Slight bubbles indicate a positive reaction due to release of oxygen.

3.6.4.2. Sugar fermentation test

Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to the bio-oxidation of a substrate, frequently a carbohydrate. Thus, different sugars were used for determining the fermentation profile and further characterization of *lactobacilli* isolates.

The sugar utilization pattern of *lactobacillus* isolates was studied using Hicarbo Kit (HiMedia). The test was performed by thawing kit at room temperature (one kit has three parts/strips ie, Part A,B,C and both A & B part contained 12 sugars, part C contains 11 sugars and a control also). Part A contained lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose.Part B contained inulin, sodium gluconate, glycerol, salicin, dulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, α-methyl-D-glucoside.Part C contained rhamnose, cellobiose, melezitose, α-methyl –D mannoside, xylitol, ONPG, esculin hyrolysis, D-arabinose, citrate utilization, Malonate utilization, Sorbose and last one was a control cupule.

Each strip was aseptically opened and peeled off the sealing foil,then inoculated with 50 µl of *lactobacillus* isolates culture whose OD was adjusted to 0.5 at 620 nm by surface inoculation method and incubated for 48 h at 37°C. Colour changes were noticed and interpretation of the results was done as per the result interpretation chart provided with the kit.

3.7. *In–vitro* evaluation of *Lactobacillus* isolates for probiotic properties

Lactobacillus isolates were evaluated by various laboratory check tests which are necessary for proving its probiotic potency.

3.7.1. Aggregation Test

This test was carried out as per the method of Reniero *et al.* (1992). The test was performed by putting the overnight culture of *lactobacillus* isolates in ependorf tubes and kept undisturbed to see the degree of aggregation of bacteria to the bottom of the tubes, leaving a clear supernatant fluid. The test tubes were examined every 15 min for 2 h.

3.7.2. Acidic pH tolerance test

This test was carried out as per the method of Garriga *et al.* (1998). Cell suspensions were prepared as detailed above in phosphate buffer at pH 2, 3 and 7.2.These suspensions were then incubated for 90 min at 37°C. Then suspensions were spread plated on MRS agar and incubated at 37°C for 48 h anaerobically to see the growth of isolates. The viable cells *ie.*, cfu were then counted and compared with the corresponding control plates incubated at pH 7.2.Thus percentage of bacteria survived was calculated.

3.7.3. Bile salts tolerance test

This test was carried out as per the method of Garriga *et al.* (1998). Overnight cultures of the isolates were centrifuged at 7,500 X g for 5 min at 4°C. After re-suspending the culture pellets in the phosphate buffer (pH 6), it was diluted 1 X 10^{-5}dilution.Subsequently, the counts of viable cells were determined by growing the suspensions on MRS agar containing different concentration of ox bile (viz. 0.15, 0.3, and 1% (wt/ vol) anaerobically at 37°C for 48 h.

3.7.4. Detection of enzymatic activity

Lactobacillus isolates were evaluated for different essential enzyme activity as mentioned below. Enzyme (protease, phytase and lipase) activities will be conducted according to Taheri *et al.* (2009). In order to detect the protease, phytase, and lipase activities, the *lactobacillus* isolates were subcultured and then spot-inoculated onto relevant medium, which were incubated anaerobically for 48 h at 37°C. After incubation, the diameter of halo zones surrounding inoculation wells were measured with a caliper.

3.7.4.1. Protease activity

For detection of protease activity, the *lactobacillus* isolates was cultured on MRS broth and after anaerobic incubation for 24 h at 37°C, 30 µl of culture supernatant was inoculated to the well punctured on medium consisting of skim milk (1%) and agar (1.5%). Diameter of clear zones surrounding each well was measured with a caliper.

3.7.4.2. Phytase activity

The MRS broth which contains 0.25% calcium phytate (Sigma- Aldrich Co., St. Louis, MO, USA) was used to sub-culture the *lactobacillus* isolates and the medium consisted of glucose (1.5%), calcium phytate (0.5%), NH_4NO_3 (0.5%), KCl (0.05%), $MgSO_4.7H_2O$ (0.05%), $MnSO_4.7H_2O$ (0.02%), $FeSO_4.7H_2O$ (0.001%) and agar (1.5%) (adjusted to pH 7.0 by Ca(OH)2) was used for detection of clear zone.

3.7.4.3. Lipase activity

For lipase activity test, the MRS broth containing olive oil (1%) and Arabic gum (1%) was used to sub-culture the *lactobacillus* isolates. Activity was detected by using a medium that consisted of tryptone (0.1%), yeast extract (0.5%), NaCl (0.05%), olive oil (0.1, 0.5 or 1%), Arabic gum (1%) and agar (1.5%).

3.7.5. Cell surface hydrophobicity

Lactobacillus isolates were grown in MRS broth (3 ml) at pH 6.0 with cysteine. After that cultures were washed with PBS buffer and resuspended as described previously.Then 2 ml of bacterial suspension was transferred into another tube and xylene (0.4 ml) will be added. Tubes were shaken for 2 min. and kept it for 15 min. After that O.D. of aqueous phase at 600 nm was measured. Hydrophobicity was calculated as the percentage decrease in the OD_{600} of the bacterial suspension due to partitioning of cells into the hydrocarbon layer. Percentage of hydrophobicity was calculated according to the following equation.

$$\%H = [(A0-A)/A0] \times 100$$

Where A0 and A, are the absorbance before and after xylene extraction respectively (Del Re *et al.*, 1998; Mishra and Prasad, 2005).

Those isolates which showed good aggregation, high pH and bile salt tolerance,high enzyme activity and high percentage of cell surface hydrophobicity were selected for further screening tests in the analysis of probiotic quality of the isolates.

3.7.6. Co-Aggregation Test

This test was carried out as per the method of Jin *et al.* (1996). Co-aggregation was done using suspensions of *lactobacillus* isolates culture, Salmonella Enteritidis and Salmonella Typhimurium in phosphate buffer at pH 7 adjusted to an OD_{600} of 0.5. A suspension (0.5 ml) of each pathogen and suspension (0.5 ml) of the *lactobacillus* isolates was placed together in a test tube and mixed thoroughly using a vortex. The OD_{600} of the bacterial mixture was measured after incubation for 4 h at 37°C. The OD of control tubes contained 1 ml of a suspension of each bacterial species was also obtained. The percentage of co-aggregation was calculated using the equation of Handley *et al.* (1987).

Formula for Percentage of co-aggregation

= {[(PC + LC) /2 “ (P + L)] / (PC + LC) /2} × 100

Where PC and LC represent the optical densities in control tubes containing only pathogen or *lactobacillus* culture after 4 hours of incubation respectively and P + L represent the optical density of the mixed culture after the same period of incubation.

3.7.7. Detection of antagonistic activity

This test was carried out as per the method of Schillinger and Lucke *et al.,*1989.For detection of inhibitory activity of *lactobacillus* isolates on pathogenic bacterial species, the well diffusion assay was followed. Plates containing solidified nutrient agar were overlaid with soft nutrient agar (0.7% agar in nutrient broth) and spread plated with *Salmonella Typhimurium, Salmonella Enteritidis* and *E.coli* cultures. Wells were made at the periphery and one at the center each 6 mm in diameter, in the agar, and 30 µl of culture supernatant of each *lactobacillus* isolates (CFS) obtained by centrifugation at 7500 rpm for 5 minutes was transferred into each well. The plates were incubated aerobically for 24 h at 37°C after which examined for clear inhibition zones around the wells.

3.8. Molecular Characterization of selected *lactobacillus* isolates

Based on above screening tests results isolates having good probiotic qualities were characterized further by molecular methods using genus specific primers. Then the PCR product was used for partial sequencing of 16SrDNA for species level identification.

3.8.1. Isolation of Genomic DNA of *lactobacillus* isolates

Genomic DNA from individual *lactobacillus* isolate collected using standard protocol of Phenol: chloroform: iso-amyl alcohol extraction method (Jian ye *et al.,* 2004).

3.8.1.1. Procedure

Inoculum of *lactobacillus* culture was poured into microfuge tubes of 2 ml capacity and centrifuged at 13000 rpm for 5 minutes. *Lactobacillius* isolates in the form of white pellet settled at the bottom of the tube and this process was repeated 2 times in order to obtain finer pellet at the bottom. Then 500 µl of lysis buffer was added to each tube and kept at 37°C for 1 hour. 10 µl of proteinase K and 50 µl of SDS was added and kept at 55°C in water bath 1 hour. 500 µl of PCI (Phenol: Chloroform: Isopropyle alcohol in the ratio of 25:24:1) was added to the tube and the tube appears milky white and later centrifuged at13000 rpm for 10 minutes. Then supernatant was taken in a fresh tube. Again PCI was added and centrifuged at 13000 rpm for 10 minutes and the clear fluid was taken in a fresh tube. Then 500 µl of chloroform was added and centrifuged at 13000 rpm for 10 minutes followed by pipetting out the supernatant into a fresh tube. Then 50 µl of sodium acetate and 500 µl of isopropyl alcohol were added and spinned at 13000 rpm for 5 minutes. Then added 100 µl of ethanol spinned at 13000 rpm for 5 minutes, Pellet dried at 37°C in an incubator for 20 minutes followed by addition of 50 µl of nuclease free water to dissolve genomic DNA.OD at 260/280 nm of individual DNA samples were checked using Nano drop-1000 (Thermo Scientific, Singapore) and recorded for quantification. Quality of individual DNA samples was checked on 1.5 % Agarose Gel Electrophoresis (AGE).The semi-dried extracted DNA was resuspended in 20µl of nuclease free water and stored at -20°C until use.

3.8.2. Polymerase chain reaction (PCR)

Good quality DNA samples were used for PCR amplification and the PCR mix and cycling conditions were optimized. Primers used for amplification targeted the region of 16S-23S r RNA Intergenic Spacer Region (ISR) for characterization of *Lactobacillus* isolates at genus level. As the sequences of the 16S/23S ribosomal RNA intergenic spacer region of *lactobacillus* are phylogenetically conserved, universal primer of lactobacillus against this sequence were used to perform polymerase chain reaction (PCR). Primer used as refered by Dubernate *et al.* (2002) (Reverse primer LbLMA1-rev 5P-CTC AAA ACT AAA CAA AGT TTC-3P and forward primer R16-1 (5P-CTT GTA CAC ACC GCC CGT CA-3P) corresponding to the terminal sequence of the 16S r RNA gene and conserved among lactobacilli was used. PCR conditions and amplification was carried out in a thermal cycler PTC 200 (Bio rad). The reaction mixture (50 µl) contained 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (Invitrogen), 1xPCR buffer without $MgCl_2$,1.0 mM $MgCl_2$, 100 ng of bacterial DNA and 2.5 U of Taq DNA Polymerase. DNA fragments were amplified as per the condition like initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a 7 min final extension step at 72°C. The products were stored at 4°C until analysis.

3.8.3. Procedure for Gel electrophoresis

5 µl aliquots of the amplified products were subjected to electrophoresis in 1.5% agarose gel in TAE buffer containing 40 mM Tris acetate and 1 mM EDTA maintained at pH 8.2. Gels were stained with ethidium bromide (5 µg /ml) and visualized under UV light. A 100 bp polymer was used as a molecular mass marker (Invitrogen). The PCR product obtained was approximately 250 bp long and encompassed the 16S-23SrRNA intespacer region.

PCR products were sequenced (partial sequencing of 16S rDNA) and its homology was analyzed through BLAST (NCBI) for species level identification of isolates. Neighbor-joining phylogenetic tree based on 16 S rRNA -23S rRNA intergenic spacer region gene sequences, showing the relationships between lab isolated *lactobacillus* strain and representative members of the genus *Lactobacillus* was prepared using MEGA-5 with Bootstrap percentages based on analysis of 1000 resampled datasets.

3.9. Antibiogram test

The best isolate was further checked for its resistance against certain antibiotics which are commonly used in veterinary therapeutics.Antibiotic sensitivity of the *lactobacillus* isolates was determined by the Kirby-Bauer disc method (Bauer *et al.*, 1966). The density of bacterial suspension was adjusted until the visible turbidity was equal to 0.5 McFarland standards .The inoculums was spreaded evenly over the entire surface of the plates contained MRS agar. Subsequently, paper discs containing the antibiotics were laid on the plates and incubated anaerobically at 37°C and growth inhibition will be examined after 24 hrs.

3.10. Growth bioassay

Based on the observations made from *in–vitro* analysis of *lactobacillus* isolates for their probiotic potency, the selected isolate was put into *in-vivo* analysis for assessing its feasibility as a potential probiotic candidate in poultry nutrition.

3.10.1.Experimental design

The feeding trial study was planned to examine the effect of feeding the selected *lactobacillus* isolate on adult guinea fowl and broilers .The birds were randomly assigned into four groups (T1 –T4) of 20 birds per group in adult female pearl variety guinea fowl maintained at Guinea fowl unit, CARI, Izatnagar and 25 birds of mixed sex per group in CARIBRO-Dhanraja maintained at Experimental Broiler Farm, CARI, Izatnagar. The experiment was conducted for a duration of 8weeks starting from 20 weeks of age to 28 weeks of age (breeder layer ration) in guinea fowl and 5weeks in broilers (0-3weeks-starter, 3-5weeks-finisher).Administration of feed supplements was done throughout the experimental period. Both the species were reared in deep litters under standard uniform managemental conditions. Basal feed compositions used in the trial was given in Table 2.

3.10.2.Feed supplements

Commercially available BMD (*bacitracin methylene disalicylate*) and prebiotic MOS were used for the feeding trial along with the isolated *Lactobacillus* from guinea fowl and *L.acidophilus* culture obtained from NCDC, Karnal.

3.10.3.Titration of selected *lactobacillus* isolate for dose standardization

Titration was done by serial dilution, plating and counting on MRS agar plates. After that the aliquots was adjusted to10^8 CFU/ml using sterile PBS.

3.10.4. Basal feed fermentation with titrated dose of lactobacillus isolate

Broiler (starter and finisher) and guinea fowl breeder layer ration were fermented with selected *Lactobacillus* isolate. 20% of daily ration for both species was autoclaved and daily inoculated with 15% of *Lactobacillus* isolate broth culture having viable count of 10^8cfu/ml and fermented at 37°c for 24 hours before added to daily ration afresh and mixed well. Different experimental dietary treatments used in the growth assay are given in Table 3.

3.10.5. Response criteria

The data in relation to various parameters as affected by different dietary supplementation in guinea fowl and broiler diet had been collected with reference to growth performance, cell mediated and humoural immunity, competitive exclusion of pathogens and histomorphological examination. Data related to carcass quality traits were also recorded in broilers.

3.10.5.1. Growth Performance

3.10.5.1.1. Body weight

In broilers body weights at 0th day, 7th day, 3rd and 5th week were recorded in the morning without offering the feed to birds. Biweekly body weight was taken in adult guinea fowls also in the same way. Body weights were taken by using platform digital balance.

3.10.5.1.2. Feed intake

A weighted quantity of respective diet was offered ad-lib daily to triplicate groups of each dietary treatment in the morning and the residue was weighed at every week end in order to arrive at weekly feed intake in broilers.

3.10.5.1.3. Feed conversion ratio

The FCR was determined for 0-1, 1-3 and 3-5 week duration during experimental period of 35 days in broilers. The weight gain was estimated by subtracting the weight at beginning from the weight at end of duration. Similarly, the feed consumed was also be measured by subtracting the total residual feed from total feed offered in particular duration. FCR was determined by dividing the feed consumed by weight gain during experimental period. The FCR in each period was determined as follows:

Table 2. Composition of basal feed used in feeding trials

Composition/100 kg feed	Broiler Starter	Broiler Finisher	Guinea fowl Breeder layer
Maize	55.5	62.425	58.5
DORB	2.14	1.55	4.335
Soyabean	30.6	20.5	19.2
Guar korm	4	4	4
RSM	4	4	4
Fish meal	0	4	0
Oyster shell	0	0	2
Marble chips	0	0.6	3.3
Limestone	0.8	0.5	2.5
DCP	2	1.6	1.4
Salt	0.3	0.3	0.3
DL-Methionine	0.1	0.07	0.05
Lysine	0.135	0.07	0
CP%	22.1586	19.711	17.7199
ME	2816.66	2878.785	2677.01
Cacium	1.06984	1.05871	3.280123
Available P	0.4906	0.4138	0.37626
Lysine	1.2408	1.0038	0.82111
Methionine	0.4907	0.4294	0.377078

(TM premix-0.1%, vit.premix- 0.15%, Bcomplex-0.015%, Ch.chloride 0.05%,Toxine bind-0.05%, Coccidiostat-0.05%, Soda.bicarb-0.5%, Composition of trace minerals includes $FeSO_4$ 80 Mg/kg of diet, $ZnSO_4$ and $CusSO_4$ 8 mg/kg diet, $MnSO_4$ 65 mg/kg diet and KI 1.2 mg/kg diet. Composition of Vitamin Premix includes Choline chloride 500 mg/kg of diet, Niacin12 mg/kg of diet, Pyridoxine hydrochloride1.6 mg/kg of diet, Vitamin A 8250 IU/ kg diet, Vitamin B10.8 mg/kg of diet, Vitamin B2 8 mg/kg of diet, Vitamin D31200 IU kg diet, Vitamin E 10 mg/kg of diet and Vitamin K 1 mg/kg of diet.)

Table 3. Different dietary treatments in the growth bioassay.

Sl. No.	**DIETARY TREATMENTS(**Throughout the experimental period)
T-1	Basal standard feed only (Control)
T-2	Basal ration + BMD @20g/100kg
T-3	Basal ration + (10^8cfu of *L.acidophilus.*/ gram fermented feed+ Prebiotics (MOS) @ 1g/kg feed
T-4	Basal ration+ (10^8cfu of lab isolated *L. plantarum* / gram fermented feed) + Prebiotics (MOS) @ 1g/kg feed

Formula for FCR = Feed consumed in g / Weight gain in g

3.10.5.2. Mortality

Daily monitoring of birds was done in both species.

3.10.5.3. Carcass traits

At the end of the study period 6 broiler birds per each dietary group were randomly elected for slaughter. At 5^{h} weeks of age, birds were starved for 12 h with access to water and then slaughtered by severing the carotid artery and jugular veins. After defeathering, the birds were eviscerated. Feet and shanks were removed at the tibio-tarsus joint and the head at the atlanto-occipital articulation. The viscera were removed as usual dressing of poultry carcasses. The heart, liver and empty skinned gizzards were weighed individually and their sum of weights giblets was taken. The following parameters were calculated as per the formula given below. The carcasses were cut into neck, wings, thighs, drumsticks, breast and back for recording the percentage of cut up parts. Cut up parts yield was calculated as the ratio between the part weight and carcass weight (eviscerated weight without giblet).

1. Pre-slaughter weight (gm)

2. Dressed yield % = $\dfrac{\text{Dressed weight with giblet (gm)}}{\text{Pre slaughter live weight (gm)}}$ x 100

3. Carcass yield % = $\dfrac{\text{Eviscerated weight without giblet/carcass weight (g)}}{\text{Pre slaughter live weight (gm)}}$ x 100

4. Heart % = $\dfrac{\text{Weight of heart (gm)}}{\text{Pre slaughter live weight (gm)}}$ x 100

5. Liver % = $\dfrac{\text{Weight of liver (gm)}}{\text{Pre slaughter live weight (gm)}}$ x 100

6. Gizzard % = $\dfrac{\text{Weight of gizzard (gm)}}{\text{Pre slaughter live weight (gm)}}$ x 100

6. $$\text{Giblet \%} = \frac{\text{Weight of heart + Liver + Gizzard (gm)}}{\text{Pre slaughter live weight (gm)}} \times 100$$

6. $$\text{Part yield \%} = \frac{\text{Weight of part (neck/breast/wings/back/thigh/drumstick) (gm)}}{\text{Carcass weight (gm)}} \times 100$$

3.10.5.4. Immune organ weights

1. $$\text{Thymus \%} = \frac{\text{Thymus weight (g)}}{\text{Pre slaughter live weight (g)}} \times 100$$

2. $$\text{Spleen \%} = \frac{\text{Spleen weight (g)}}{\text{Pre slaughter live weight (g)}} \times 100$$

3. $$\text{Bursa of fabricius\%} = \frac{\text{Bursa of of fabricius weight (g)}}{\text{Pre slaughter live weight (g)}} \times 100$$

3.10.5.5. GI tract microflora population

GIT contents were collected separately from crop, ileum ad caeca at the end of experiment from guinea fowl and broilers for bacterial enumeration *viz Lactobacilli*, Salmonella and *E. coli.* GI tract contents were diluted 10-fold with buffered peptone water and vortexed for 2 min; 100 microliters of supernatant was smeared onto appropriate selective media in duplicate plates and incubated under optimum time-temperature combinations. After incubation period bacterial counts were recorded as cfu/ml.

3.10.5.6. Immune response

At the end of experiment, 8 birds were randomly picked up from each of the four groups and were examined for immunological parameters. It is pertinent to examine the effect, if any of supplementing ingredients to Guinea fowl/broiler, on their immunological status against New Castle disease vaccine.

3.10.5.7.1. Humoral immune response- Haemagglutination inhibition (HI) test

The humoral immune response was studied by estimating the RD-HI antibody titres by Haemagglutination inhibition test. The birds were immunized with RDF-1 (broilers-1 drop oronasally) / R_2B (guinea fowl- i/m route-0.5 ml dose) strain at 15th day of experiment and at 21st day of immunization., blood from jugular vein was collected aseptically for sera collection from 8 birds /dietary group and following test was run.

3.10.5.6.1.1. Procedure for Haemagglutination (HA) test

This method was devised by Allan and Gough (1974). Two fold serial dilution of the virus samples were prepared in NSS in ‘U’ bottom wells of microtitre plates in 50 µl volumes. 50 µl of 1% RBC (Red blood cells) suspension was added to each virus dilution along with RBC’s control wells. Contents were mixed by gently agitating the plate and incubated at room temperature for 30 min. or until a clean pattern of haemagglutination was appeared. The end point was taken as the well in which there was a positive pattern of agglutinated RBCs. Negative results and RBC control gave button appearance at the bottom of the well. HA titre was determined as the reciprocal of the highest virus dilution giving positive pattern of agglutination.

3.10.5.6.1.2. Preparation of the working haemagglutinin (4 HA):

The RD F1 vaccine was removed from deep freezer, thawed and diluted to give the required 4 HA units as per the result of HA titre.

3.10.5.6.1.3. Procedure for Preparation of red blood cells:

Blood from two healthy chickens was taken by hypodermic syringe into heparin solution (20 IU/ml). The RBCs were washed three times by light centrifugation in NSS at 1500 rpm for 10 minutes. 1% chicken red blood cell suspension was then made by mixing 1 ml of gravity deposited RBC with 99 ml of NSS.

3.10.5.6.1.4. Procedure for Haemagglutination inhibition (HI) test:

This method was devised by Allan *et al.* (1974). Two fold serial dilutions of sera were made in NSS in ‘U’ bottomed microtitre plates. The 4HA units of RD virus in equal volume (25 µl) were added to each serum dilution and incubated at 37°C for 45 min. Thereafter, 1% chicken RBC in 25 µl volume was added to each well and incubated at 37°C for 15 min. The HI titre was taken as the reciprocal of the highest dilution of serum showing complete inhibition of agglutination of the RBCs.

3.10.5.6.2. Cell mediated immune response (CMI)

Lymphocyte proliferation assay using MTT (3-4,5-dimethyl thiazole 2,5-diphenyl tetrazolium bromide) to estimate the CMI response to ND vaccine.

The MTT calorimetric assay for cellular proliferation described by Bounous *et al*. (1992) was followed with necessary modifications.

Peripheral blood mononuclear cells were collected at 21st day postvaccination and assessed for ND vaccine (RD-F/R_2B) specific CMI immune response. Whole blood sample from experimental as well as control birds were drawn aseptically from jugular vein into sterile syringes rinsed with heparin & collected in tubes containing heparin 20IU/ml of blood. Tubes were centrifuged at 1500 rpm for 20 min, and buffycoat was taken and overlaid onto Histopaque1.077 density gradient medium (Sigma) and centrifuged at 1900 rpm for 20 min. Lymphocytes at the interphase were collected, washed three times in RPMI-1640 (colorless, without indicator) and resuspended in RPMI-1640 supplemented with 10% FBS medium.For proliferation assays, 50 µl of cell suspension in RPMI-1640 was placed into two sets of triplicate wells ($2x10^6$ viable cells/well) of 96- well flat-bottomed tissue culture plates (Nunclone). The first set of triplicate wells received 50µl of RPMI-1640 with 10% FBS and second set received 50 µl of RPMI-1640 with 10% FBS and Concanvalin A(Con A,Sigma) having stock of 5 µg/ml was added to each well (50 µl/well). Plates were incubated at 37^0C in a humidified incubator under 5% CO_2. 20 µl MTT (from 5 mg/ml stock) was added to each well after 72 h of incubation. The plates were further incubated for 4 h at 37^oC under 5% CO_2 and then 120 µl DMSO was added to each well. After mixing the dark precipitate by pipetting the plate was read at 510 nm with reference wavelength of 650 nm using a microtitre plate reader (BioRad, USA).

The stimulation Index (SI) was calculated by the following formula :

SI= (Mean OD of Con- A stimulated cell / (Mean OD of unstimulated cells)

3.10.5.7. Intestinal histomorphology

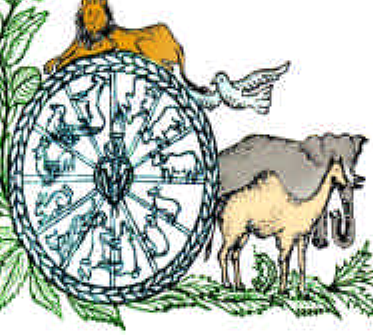
The effect of feeding supplements on the morphology of the vital organ *ie.*, intestine in guinea fowl and broilers was examined. At the end of experiment, birds were sacrificed and sections of intestine were cut and slides were prepared and examined using an optical microscope (Chiou *et al.,* 1999). The villus height, crypt depth, and villus height crypt depth ratio measured. Sample collected from duodenum, ileum of guinea fowl and broiler birds were processed for histomorphological study. Histological technique involved processes like Fixation of tissue, Dehydration, Clearing, Embedding ,Cutting and Staining. Fixation in 10% formalin with approximately 10-20 times the volume of the specimen was done. Tissues were dehydrated by using increasing strength of alcohol like 50%, 70%, 90% and 100%. Clearing was done by replacing alcohol by xylene for 0.5–1 hour. Impregnation of tissue with Wax was done at melting point temperature of paraffin wax and the volume of wax was about 25-30 times the volume of tissues for a total duration of 4 hours. Impregnated tissues were placed in a mould with their labels and then fresh melted wax was poured in it and allowed to settle and solidify. Then section cutting was done followed by staining for histomorphological examination of duodenum and ileum. Histomorphological examination was done in terms of measurement of parameters like villous height, cryptal depth and villous crypt ratio of 6 birds each of different dietary treatments.

3.11. Statistical analysis

The data collected under various *in-vitro* probiotic quality screening tests for *Lactobacillus* isolates and *in-vivo* growth bioassay experiments for the supplemental effect of selected *lactobacillus* isolate were statistically analyzed (SPSS 20). Differences among groups were determined using Duncan’s multiple-range test. Statements of statistical significance were based on P<0.05. The reads obtained in metagenomic analysis of crop microbiome of guinea fowl were analysed using different programs viz. Greengenes, LSU, RDP, SSU, MG-RAST, Krona etc.

* * *

Results and Discussion



4

RESULTS AND DISCUSSION

The present study comprised of collection of intestinal contents from crop, proventriculus, ileum and caecum of Guinea fowl (pearl variety) for *lactobacillus* isolation, identification, characterization and screening for probiotic potency at species level. The probiotic potency evaluation was carried out by various screening tests which included aggregation, tolerance to bile salts and acidic conditions, enzymatic activities, cell surface hydrophobicity, co-aggregation, antagonistic tests and antibiotic sensitivity tests. Evaluated *lactobacillus* isolate was utilized for feed fermentation and subsequently for growth bioassay study in guinea fowl and broiler (CARI Dhanraja). In growth bioassays study, different feed supplements *viz;* lab isolated *lactobacillus* species, *Lactobacillus acidophilus*, prebiotic MOS and antibiotic BMD were used. During growth bioassay, the performance of broilers in respect of body growth, feed intake, feed conversion, carcass traits and survivability were investigated up to 5 weeks of age while in guinea fowl body growth and survivability were investigated from 22-28 weeks of age. The immune response of experimental guinea fowl and broilers subjected to different dietary treatments was also investigated in terms of humoral immunity and cell mediated immunity at 21st day of vaccination against RD. At the end of experiment histomorphometry of small intestine was performed to visualize any changes in villi height and crypt depth due to dietary treatment in both species and checked competitive exclusion property of the isolate against *E.coli* and Salmonella. Data on such observations were analyzed statistically for treatment effects and presented/ discussed appropriately under relevant portions in this chapter.

4.1. Metagenomic analysis

DNA sample obtained from crop of adult guinea fowl was analyzed using Next Generation Sequencing (NGS) and bio-informatics tools to obtain a picture of crop microbiome in guinea fowl which may help to carry out intended modulation by feed supplementation of probiotic as an alternative to antibiotic growth promoters. DNA from crop sample was isolated and the quality control analysis was performed using Nanodrop (77.9 ng/ml), qubit fluorometer (66.3 ng/ml) and gel electrophoresis. Then the amplicons were generated using specific primers from different hypervariable regions of 16S rRNA. The prepared libraries were quantified using Qubit fluorometer and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip. The libraries were subjected for NGS on Myseq (Illumina).The reads generated were analyzed using different programs viz. Greengenes, LSU, RDP, SSU, MG-RAST, Krona etc. Source hit distribution from different databases are given in Fig.1.Phylogenetic tree generated for crop sample at the level of order using the annotation information obtained through M5NR database is given in Fig.2.

The metagenomic results revealed domains distributed as 0.0026% Archaea, 59.94% Bacteria, 8.65% Eukaryota, 31.17% Viruses and 0.23% others in the crop microbiome of guinea fowl (Fig.3). Taxonomic distribution under phylum showed Firmicutes 44.02%, unclassified microbial genome derived from viruses 31.17%, Chordata 7.98%, Proteobacteria 6.04%, unclassified microbial genome derived from bacteria 4.64%, Bacteriodetes 3.37%, Actinobacteria 1.34%, Arthropoda 0.3%, Streptophyta 0.26% and Tenericutes 0.18% (Fig.4). Taxonomic distribution under class included unclassified microbial genome derived from viruses 31.17%, Clostridia 25.89%, Bacilli 16.81%, unclassified microbial genome derived from bacteria 4.64%, Aves 4.2%, epsilon Proteobacteria 2.07%, Mammalia 1.91%, beta Proteobacteria1.79%, Bacteioidia 1.74% and Flavobacteria1 49% (Fig.5).

Major orders found in crop microbiome were unclassified microbial genome derived from viruses 31.17 %, Clostridiales 25.83%, Lactobacillales16.18%, unclassified microbial genome derived from bacteria 4.64%, Galliformes 3.49%, Campylobacteriales 2.07%, Burkholderiales 1.77%, Bacteriodales 1.74%, Flavobacteriales 1.48%, Selenomonadales 1.23% (Fig. 6).

Data showed that crop of guinea fowl contained important families like Microviridae 31.01 %, Clostridiaceae 21.42%, Lactobacillaceae 14.76%, unclassified (derived from bacteria) 4.63%, Phasianidae 3.45%, Campylobacteraceae 1.71%, Flavobacteriaceae 1.48%, Ruminococcaceae 1.4%, Bacteroidaceae 1.38%, Veillonellaceae

Fig. 1: The graph displays the number of features in examined dataset that were annotated by the different databases. The bars represent annotated reads, which are colored according to their e-value range.

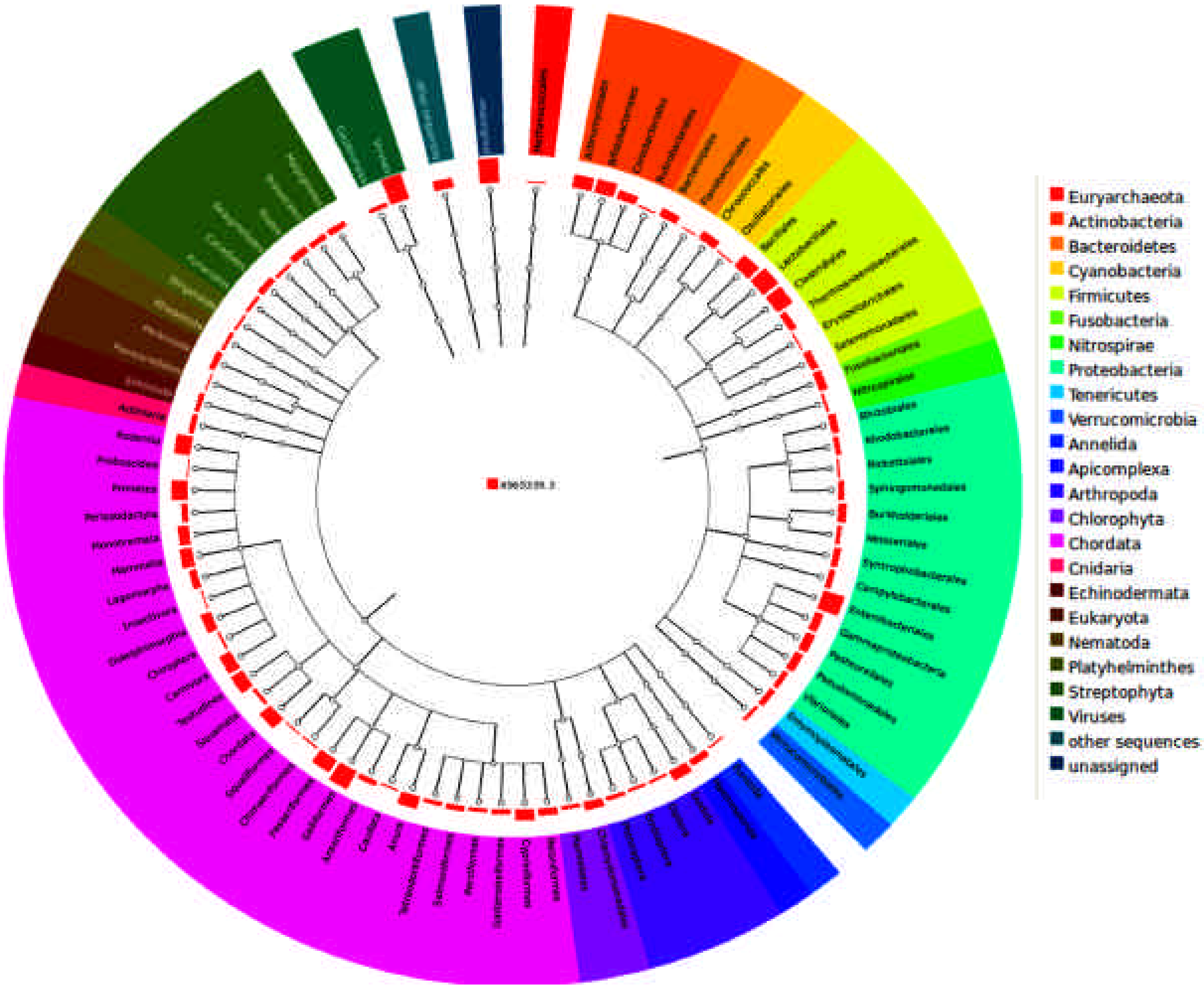


Fig. 2: Phylogenetic tree generated for crop sample at the level of order using the annotation information obtained through M5NR database.

Table 4. Taxonomic distribution of crop microbiome in guinea fowl

Domains	% of abundance	Phyla	% of abundance	Classes	% of abundance	Orders	% of abundance	Families	% of abundance	Genuses	% of abundance
Archaea	**0.0026**	Firmicutes	**44.02**	unclassified (derived from Viruses)	**31.17**	unclassified from viruses	**31.07**	Microviridae	**31.01**	Microvirus	**30.98**
		unclassified (derived from Viruses)	**31.17**	Clostridia	**25.89**	Clostridiales	**25.83**	Clostridiaceae	**21.42**	Lactobacillus	**14.64**
Bacteria	**59.94**	Chordata	**7.98**	Bacilli	**16.81**	Lactobacillales	**16.18**	Lactobacillaceae	**14.76**	Candidatus Arthromitus	**11.00**
		Proteobacteria	**6.04**	unclassified (derived from Bacteria)	**4.64**	unclassified from bacteria	**4.64**	unclassified (derived from Bacteria)	**4.63**	Clostridium	**10.36**
Eukaryota	**8.65**	unclassified (derived from Bacteria)	**4.64**	Aves	**4.20**	Galliformes	**3.49**	Phasianidae	**3.45**	unclassified (derived from Bacteria)	**4.63**
		Bacteroidetes	**3.37**	Epsilonproteobacteria	**2.07**	Campylobacterales	**2.07**	Campylobacteraceae	**1.71**	Gallus	**3.38**
Viruses	**31.17**	Actinobacteria	**1.34**	Mammalia	**1.91**	Burkholderiales	**1.77**	Flavobacteriaceae	**1.48**	Campylobacter	**1.71**
		Arthropoda	**0.30**	Betaproteobacteria	**1.79**	Bacteroidales	**1.74**	Ruminococcaceae	**1.40**	Bacteroides	**1.38**
Others	**0.23**	Streptophyta	**0.26**	Bacteroidia	**1.74**	Flavobacteriales	**1.48**	Bacteroidaceae	**1.38**	Faecalibacterium	**1.23**
		Tenericutes	**0.18**	Flavobacteriia	**1.49**	Selenomonadales	**1.23**	Veillonellaceae	**1.22**	Ralstonia	**1.05**

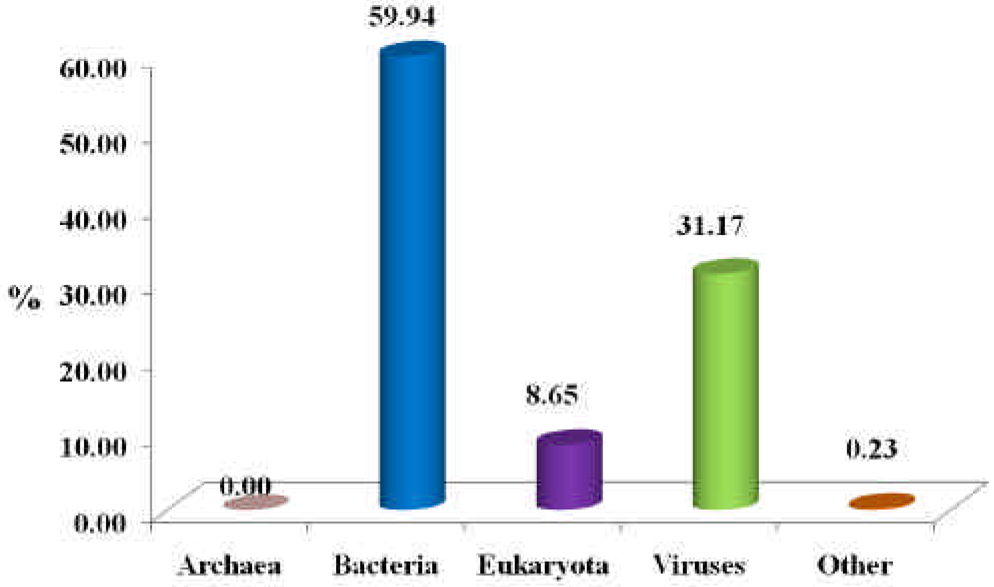


Fig. 3: Distribution of Domains in crop microbiome of guinea fowl

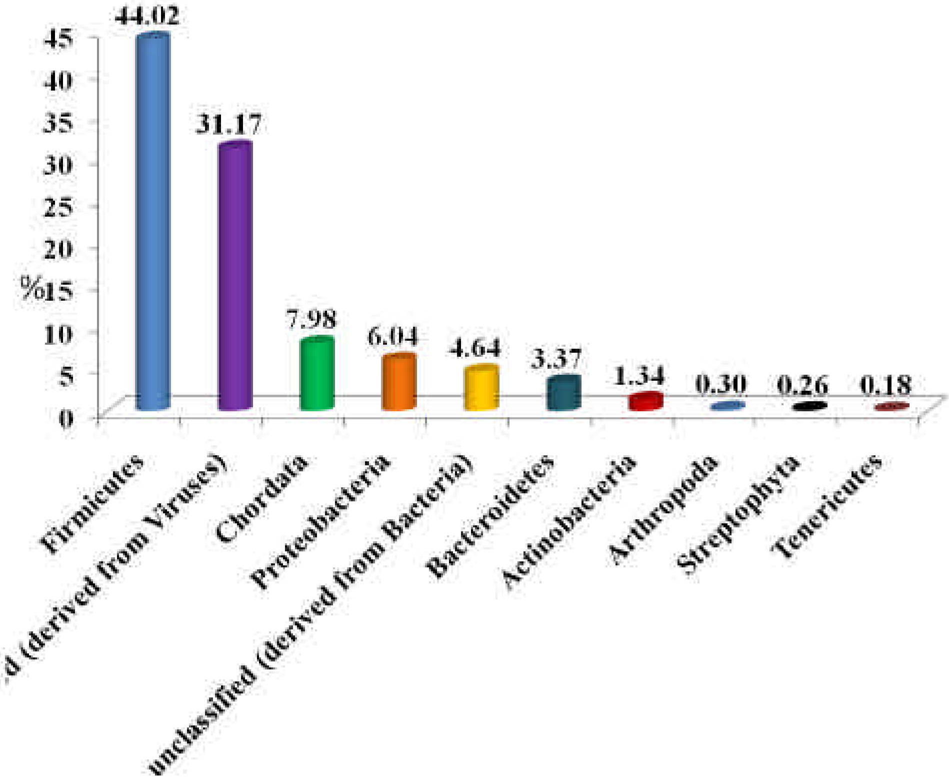


Fig. 4: Distribution of most abundant 10 Phyla in crop microbiome of guinea fowl

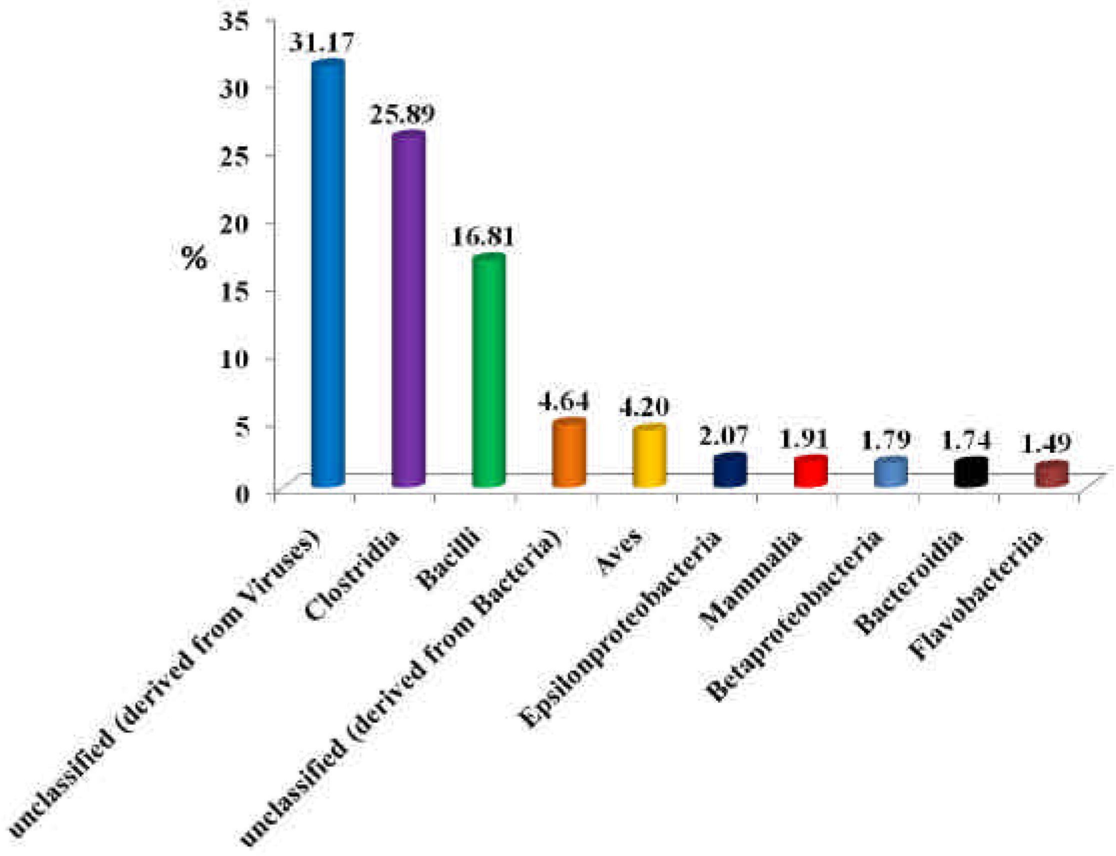


Fig. 5: Distribution of most abundant 10 Classes in crop microbiome of guinea fowl

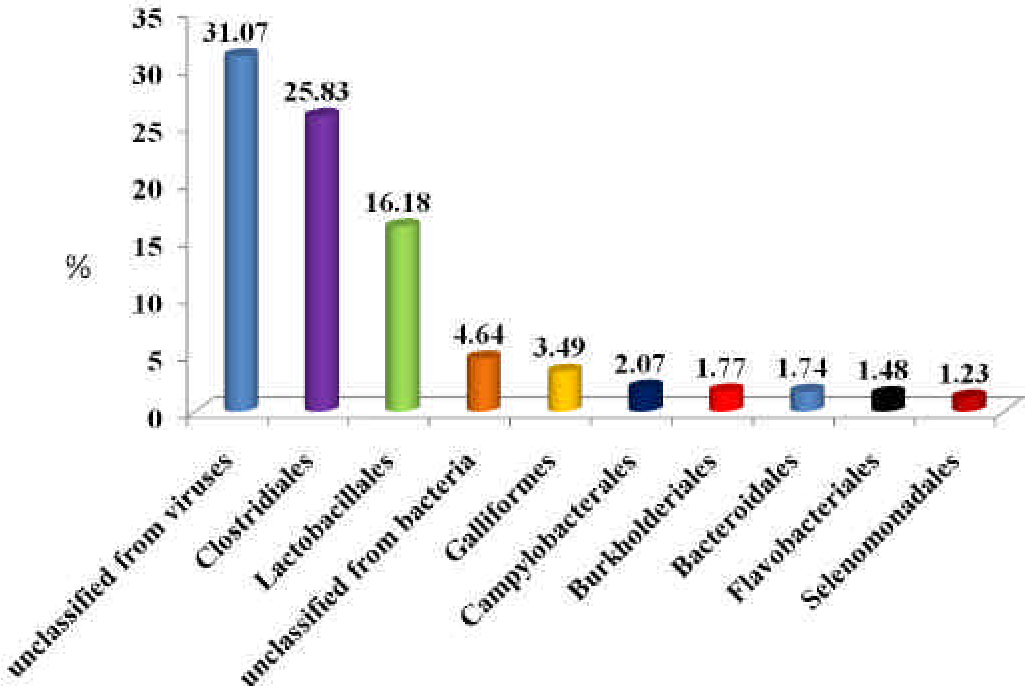


Fig. 6: Distribution of most abundant 10 Orders in crop microbiome of guinea fowl

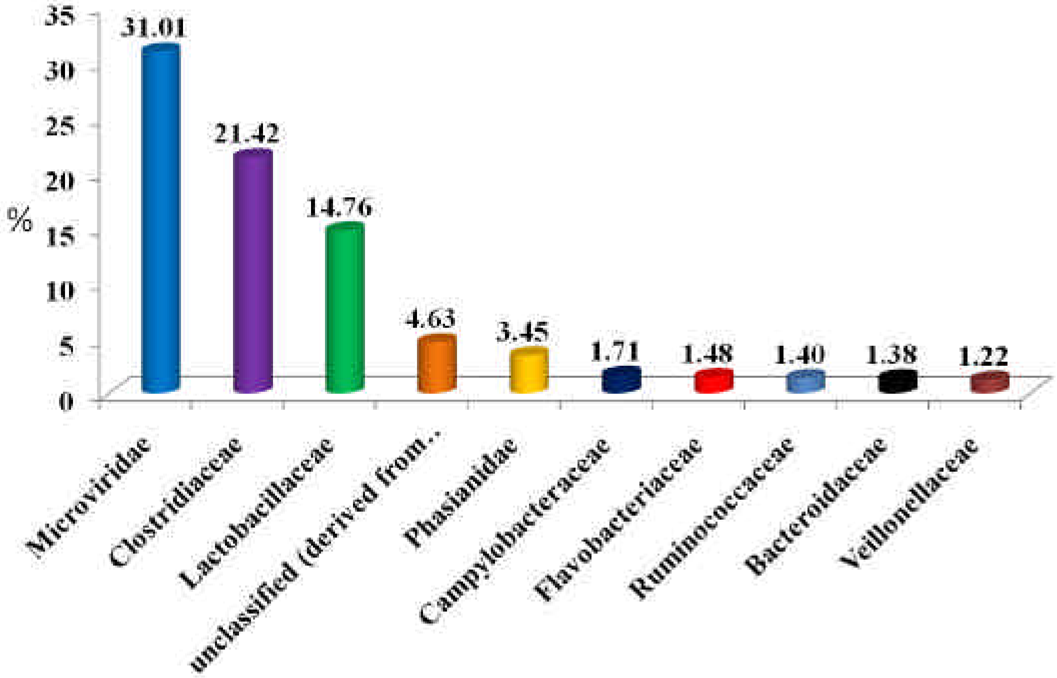


Fig. 7: Distribution of most abundant 10 Families in crop microbiome of guinea fowl

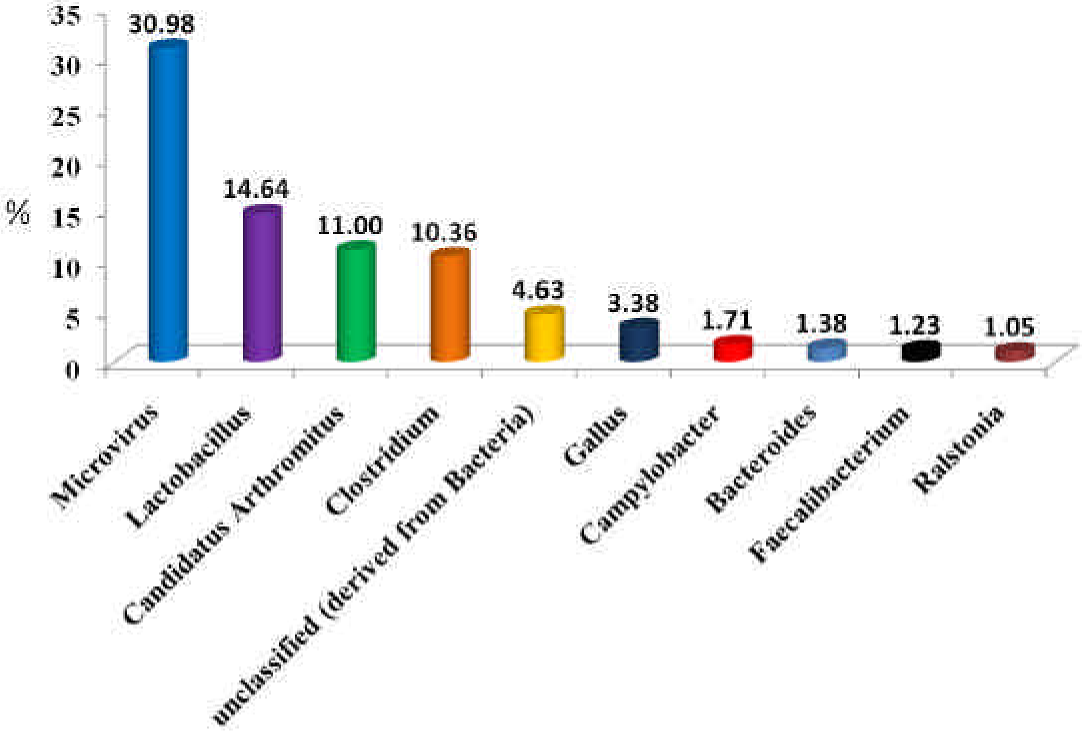


Fig. 8: Distribution of most abundant 10 Genuses in crop microbiome of guinea fowl

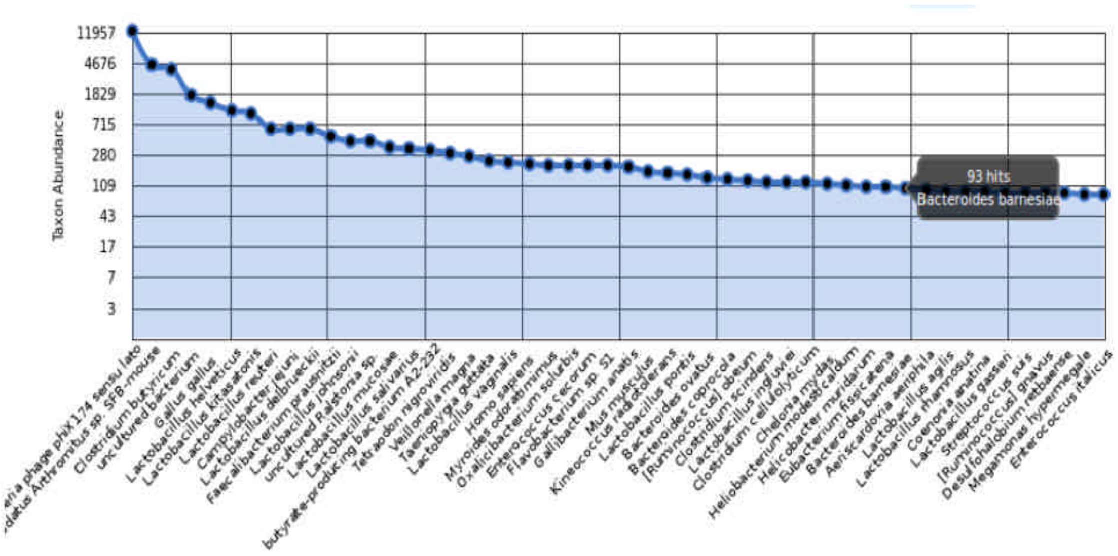


Fig. 9: Distribution of most abundant 50 Species in crop microbiome of guinea fowl

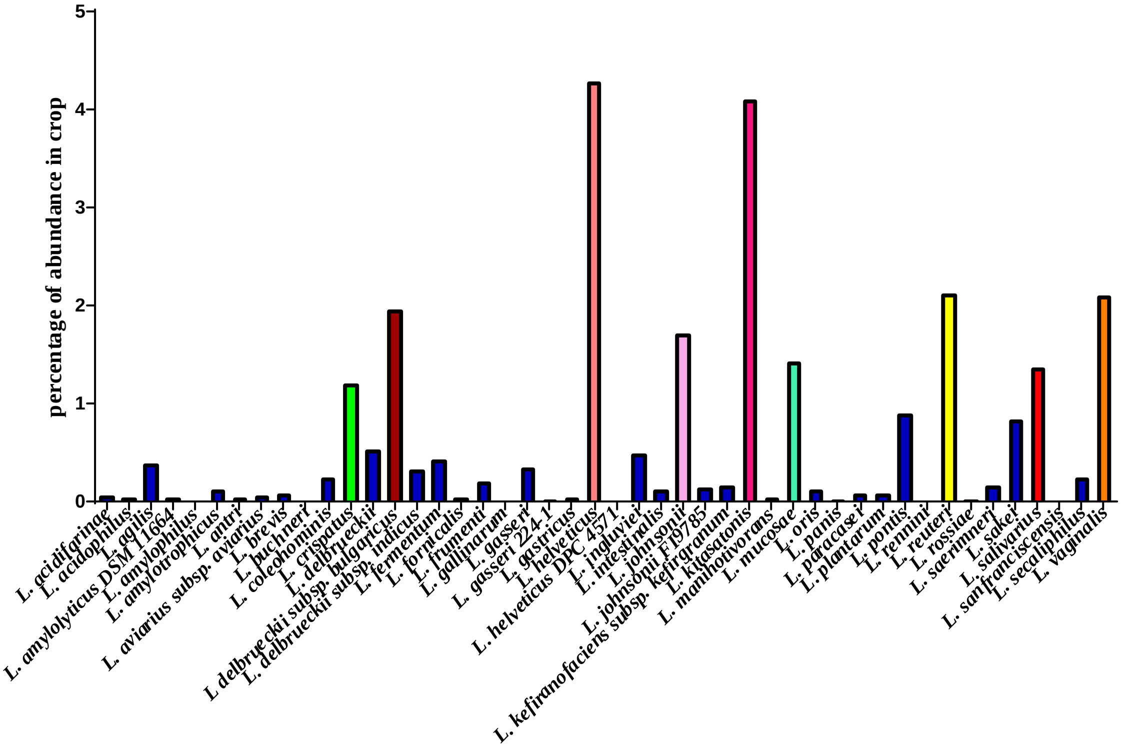


Fig. . Distribution of *Lactobacillus* strains in crop microbiome of guinea fowl

Table 5. Abundance of *Lactobacillus* strains in crop microbiome of Guinea fowl

***Lactobacillus* strains**	**Percentage of**						
	Lactobacillus	**Lactobacillaceae**	**Lactobacilales**	**Bacilli**	**Firmicutes**	**Bacteria**	**All Domains**
L. acidifarinae	0.16	0.16	0.14	0.14	0.05	0.04	0.04
L. acidophilus	0.12	0.12	0.11	0.11	0.04	0.03	0.03
L. agilis	1.61	1.60	1.46	1.41	0.54	0.39	0.38
L. amylolyticus DSM 11664	0.14	0.14	0.13	0.12	0.05	0.03	0.03
L. amylophilus	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. amylotrophicus	0.44	0.44	0.40	0.39	0.15	0.11	0.11
L. antri	0.14	0.14	0.13	0.12	0.05	0.03	0.03
L. aviarius subsp. aviarius	0.23	0.23	0.21	0.20	0.08	0.06	0.05
L. brevis	0.25	0.25	0.22	0.22	0.08	0.06	0.06
L. buchneri	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. coleohominis	0.97	0.97	0.88	0.85	0.32	0.24	0.23
L. crispatus	4.92	4.88	4.46	4.29	1.64	1.20	1.18
L. delbrueckii	2.14	2.13	1.94	1.87	0.71	0.52	0.51
L delbrueckii ssp. bulgaricus	8.11	8.05	7.35	7.08	2.70	1.98	1.94
L. delbrueckii ssp. indicus	1.33	1.32	1.20	1.16	0.44	0.32	0.32
L. fermentum	1.70	1.69	1.54	1.48	0.57	0.42	0.41
L. fornicalis	0.14	0.14	0.13	0.12	0.05	0.03	0.03
L. frumenti	0.81	0.81	0.74	0.71	0.27	0.20	0.19
L. gallinarum	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. gasseri	1.36	1.35	1.24	1.19	0.45	0.33	0.33
L. gasseri 224-1	0.04	0.04	0.03	0.03	0.01	0.01	0.01
L. gastricus	0.07	0.07	0.06	0.06	0.02	0.02	0.02

contd...

L. helveticus	17.79	17.66	16.12	15.53	5.93	4.35	4.25
L. helveticus DPC 4571	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. ingluviei	2.02	2.00	1.83	1.76	0.67	0.49	0.48
L. intestinalis	0.48	0.47	0.43	0.42	0.16	0.12	0.11
L. johnsonii	7.06	7.01	6.40	6.16	2.35	1.73	1.69
L. johnsonii FI9785	0.53	0.53	0.48	0.46	0.18	0.13	0.13
L. kefiranofaciens ssp. Kefirgranum	0.64	0.63	0.58	0.56	0.21	0.16	0.15
L. kitasatonis	17.04	16.92	15.45	14.88	5.68	4.17	4.07
L. manihotivorans	0.12	0.12	0.11	0.11	0.04	0.03	0.03
L. mucosae	5.91	5.87	5.36	5.16	1.97	1.45	1.41
L. oris	0.46	0.46	0.42	0.40	0.15	0.11	0.11
L. panis	0.05	0.05	0.05	0.05	0.02	0.01	0.01
L. paracasei	0.25	0.25	0.22	0.22	0.08	0.06	0.06
L. plantarum	0.27	0.26	0.24	0.23	0.09	0.06	0.06
L. pontis	3.73	3.71	3.38	3.26	1.24	0.91	0.89
L. rennin	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. reuteri	8.80	8.73	7.97	7.68	2.93	2.15	2.10
L. rossiae	0.04	0.04	0.03	0.03	0.01	0.01	0.01
L. saerimneri	0.57	0.56	0.51	0.49	0.19	0.14	0.14
L. sakei	3.42	3.39	3.10	2.98	1.14	0.84	0.82
L. salivarius	5.63	5.59	5.10	4.91	1.88	1.38	1.34
L. sanfranciscensis	0.02	0.02	0.02	0.02	0.01	0.00	0.00
L. secaliphilus	0.96	0.95	0.87	0.83	0.32	0.23	0.23
L. vaginalis	8.74	8.68	7.92	7.63	2.91	2.14	2.09

1.22% (Fig.7). Most abundant ten genuses include Microvirus 30.97%, Lactobacillus 14.64%, Candidatus Arthromitus 10.99%, Clostridium 10.36%, unclassified (derived from Bacteria) 4.63%, Gallus 3.38%, Campylobacter 1.71%, Bacteroides1.37%, Faecalibacterium 1.23% and Ralstonia1.05% (Fig.8). Most abundant 50 species of bacteria in crop of guinea fowl is given in Fig.9. Taxonomic distribution data are also shown in Table 4.

Around 43 species of *lactobacillus* were observed in crop of guinea fowl with a number of strains under each species through metagenomic analysis. Out of all *L. kitasatonis* and *L. helveticus* was present in highest percentage followed by *L.reuteri ,L.vaginalis, L.dulbreuki subsp. Bulgaricus and L. johnsonii, L. mucosae,, L. salivarius and L.crispatus*.Fig.10 and Table 5 shows the percentage of abundance of *Lactobacillus* strains as a part of genus *Lactobacillus*, family Lactobacilaceae, order Lactobacillales, class Bacilli, phylum Firmicutes, domain Bacteria and whole domains.

Since the bacterial populations of crop in guinea fowl showed very low population of bacteria (59.94%) with 44.02% firmicutes, as per the metagenomic data, supplementation of feed with probiotic bacteria may improve the feed efficiency and growth rate in guinea fowl.Microbial population of gastrointestinal tract are specific and particular for different animals, isolation of *Lactobacillus sp.* from Guinea fowl was carried out towards developing a specific probiotic for guinea fowl and other poultry species. The objective of this study was to isolate, characterize, and further select the most suitable strain of *Lactobacillus* from the crop, proventriculus, ileum, and caecal contents of guinea fowl through various *in vitro* probiotic quality assessment tests and to validate its potential as a probiotic poultry feed supplement through *in vivo* application.

4.2. Isolation and purification of *Lactobacillus*

Samples obtained from different segments of gut were used for isolation of *lactobacillus* in MRS broth and MRS agar (Fig 11.a,b). In many previous studies MRS has been used as selective media for *lactobacillus* isolation (Taheri *et al.,* 2009; Idoui *et al.,* 2012; Pascual *et al.,* 2009). Among several colonies developed on MRS agar plates, 32 isolates based on colonial morphology (i.e., color, size, margin and shape of the colony such as white, greyish white ,creamy or creamy white color, size varying from 0.5–2.3 mm in diameters, with entire or undulate margins) were picked up after plating 1 ml of sample that was previously enriched in 10 ml of *lactobacillus* selection MRS broth for 24 h at 37°C. Subculturing of isolates was done three times in MRS broth by incubating at 37°C for 24–48 h under 5% CO_2 and anaerobic condition. From the third subculture, a loopful of culture was streaked on the solidified Petri plates of *lactobacillus* selection MRS agar with inoculating loop incubated under standard conditions and single isolated colony was picked up from each plate and subcultured twice in MRS broth and 50% glycerol stock was made for thirty two isolates and stored at -80°C for further identification and characterization based on different morphological, physiological, biochemical and molecular methods used routinely in microbial taxonomy.

4.3. Identification of *lactobacillus* from guinea fowl GI tract

Lactobacillus isolates were phenotypically appeared as gram positive violet rod shaped /coccobacillary bacteria in the smear (Fig.12). Physiologically isolates are characterized by checking the growth at 15°C and 45°C and at different levels of NaCl in media *viz.,* 2%, 4%, and 6.5%. Some of the isolates showed growth even at low temperature 15°C and as high as 45°C. Most of the isolates showed growth at 2% and 4% NaCl concentration while 6.5% NaCl conce. inhibited growth of most of the isolates (Table 6). Biochemically in catalase test there was no bubble formation in the slide upon addition of hydrogen peroxide to isolates so, they appeared as catalase negative as indicated in Bergey’s Manual of Determinative Bacteriology for *lactobacillus* sp. (Fig.13) and the results were also supported by previous workers Taheri *et al.* (2009), Heravi *et al.* (2011), Yamazaki et *al.* (2012). Different isolates showed different types of sugar utilization patterns for 35 sugars under our study as given in Table 7, where some cupules containing sugars showed colour changes on incubation with culture containing *Lactobacillus* isolates indicating the sugar utilization by the isolates (positive tests) and in negative tests color of the cupules did not change which shows that those isolates were not able to utilize respective sugars (Fig.14 a,b&c).

4.4. Probiotic properties evaluation

After conformation of *lactobacillus* isolates at morphological, physiological and biochemical levels, they were subjected to various screening tests for evaluation

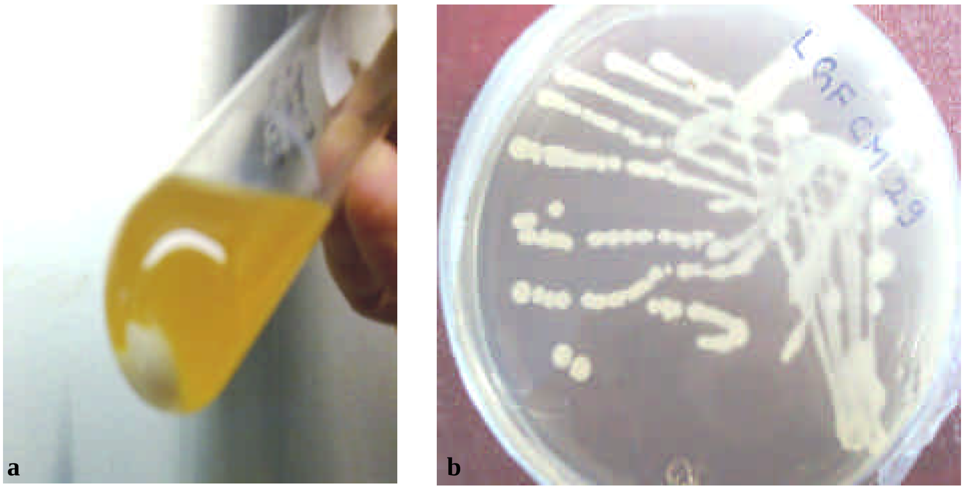


Fig. 11 a,b: **Culturing of *lactobacillus* isolates in MRS broth and MRS agar respectively**

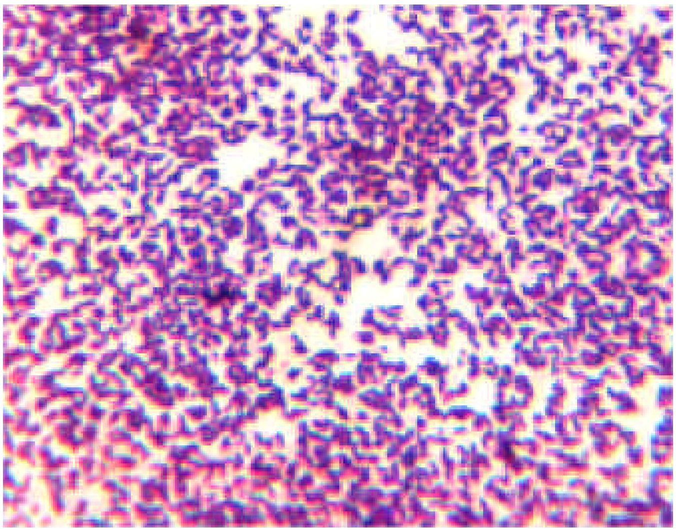
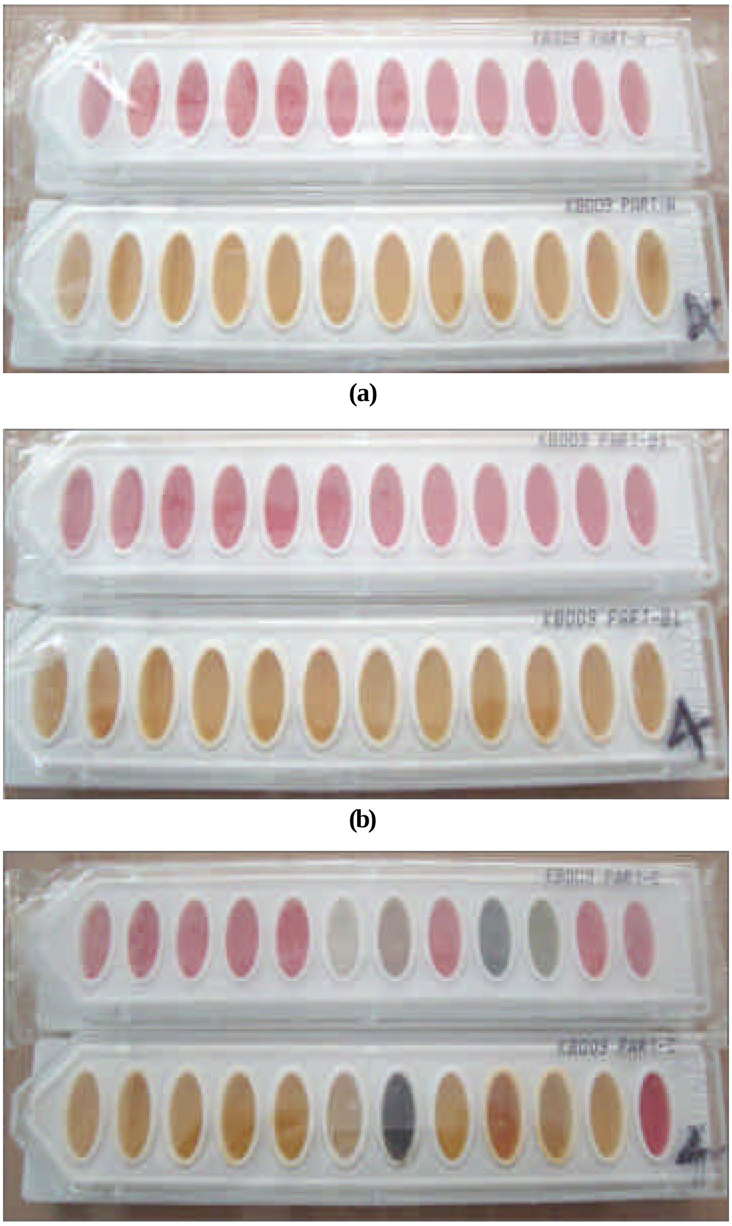


Fig. 12: Gram staining of *Lactobacillus* isolate showing Gram positive bacilli

Fig. 13: Catalase test for *Lactobacillus* isolate



(a)

(b)

(c)

Fig. 14 a,b,c: Sugar fermentation pattern of *Lactobacillus* isolate LGFCP4 in Part A, Part B, Part C of kit respectively

Table 6. Physiological characterization of *Lactobacillus* isolates

Isolates	Growth at different salt (NaCl) concentrations			Growth at different	
	Temperatures				
	2%	4%	6.5%	15°C	45°C
LGFCP1	+	-	-	-	+
LGFCP2	+	+	+	-	+
LGFCP3	+	+	+	-	+
LGFCP4	+	+	+	-	+
LGFCP5	-	-	-	-	+
LGFCP6	+	+	+	-	+
LGFCP7	+	+	-	-	+
LGFCP8	-	-	-	-	+
LGFP9	-	-	-	-	+
LGFP10	+	+	+	-	+
LGFP11	+	+	-	-	+
LGFP12	+	+	+	+	+
LGFP13	+	+	+	+	+
LGFP14	+	+	-	-	+
LGFP15	+	+	+	-	+
LGFP16	+	+	+	-	+
LGFI 17	+	+	-	-	+
LGFI 18	+	+	-	+	+
LGFI 19	-	-	-	-	+
LGFI 20	+	-	-	-	+
LGFI 21	+	+	-	-	+
LGFI 22	+	+	-	-	+
LGFI23	+	+	-	-	+
LGFI24	-	-	-	-	+
LGFCM25	+	+	-	+	+
LGFCM26	+	-	-	+	+
LGFCM27	+	+	-	+	+
LGFCM28	+	+	-	+	+
LGFCM29	+	+	-	+	+
LGFCM30	+	+	-	+	+
LGFCM31	+	+	-	+	+
LGFCM32	+	+	-	-	+

+ : Positive for growth; **- :** No growth observed

Table 7. Biochemical characterization of *Lactobacillus* isolates-Sugar fermentation tests

Lactobacillus isolates LGFCP1-LGFCM32	Sugars under study –Total 35 sugars																																		
		Xylose	Maltose	Fructose	Dextrose	Galactose	Raffinose	Trehalose	Melibiose	Sucrose	L-Arabinose	Mannose	Inulin	Sodium gluconate	Glycerol	Salicin	Dulcitol	Inositol	Sorbitol	Mannitol	Adonitol	Arabitol	Erythritol	a -methyll-D-glucoside	Rhamnose	Cellobiose	Melezitose	a -methyll-D-mannoside	Xylitol	ONPG	Esculin hydrolysis	D-arabinose	Citrate utilization	Malonate	Sorbose
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
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7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
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10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
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18	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
24	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
25	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
26	-	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	+
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	-	-	+	-	-	+
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
29	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
30	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-	-	+
31	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+

+ : Positive; **– :** Negative

Table 8. *Lactobacillus* isolates observed for their aggregation property

Isolates	**Time taken by the isolates to aggregate to the bottom of the tube (examined in every 15 min. for maximum of 2 hrs)**							
	within 15 min.	**30min.**	**45min.**	**60 min.**	**1hr 15 min.**	**1hr 30 min.**	**1hr 45 min.**	**2hrs**
LGFCP1	X	X	x	x	x	x	x	x
LGFCP2	X	X	x	x	x	x	x	x
LGFCP3	X	X	x	x	x	x	x	x
LGFCP4	*	*	*	*	*	*	complete	
LGFCP5	*	*	*	*	*	complete		
LGFCP6	**	**	**	complete				
LGFCP7	**	**	**	complete				
LGFCP8	X	X	x	x	x	x	x	x
LGFP9	X	X	x	x	x	x	x	x
LGFP10	X	X	x	x	x	*	*	*
LGFP11	X	X	x	x	x	*	*	*
LGFP12	X	X	x	x	x	x	x	x
LGFP13	x	X	x	x	x	x	x	x
LGFP14	x	*	*	*	*	*	*	*
LGFP15	***	***	complete					
LGFP16	*	*	*	*	*	*	*	*
LGFI 17	x	X	x	x	*	*	*	*
LGFI 18	x	X	x	x	x	x	x	x
LGFI 19	x	X	x	x	*	*	*	*
LGFI 20	x	X	x	*	*	*	*	*
LGFI 21	x	X	*	*	*	*	*	*
LGFI 22	x	X	x	x	x	x	x	x
LGFI23	*	*	*	**	**	**	**	**
LGFI24	x	X	x	x	*	*	*	*
LGFCM25	*	*	*	**	**	**	**	**
LGFCM26	x	X	x	x	x	x	x	x
LGFCM27	*	**	complete					
LGFCM28	x	X	x	*	*	*	*	*
LGFCM29	x	X	x	*	*	*	*	*
LGFCM30	x	X	x	*	*	*	*	*
LGFCM31	**	**	complete					
LGFCM32	x	X	x	x	x	x	x	x

*** -shows very good aggregation property,**-good ,*-Moderate, X-no aggregation observed of probiotic properties. Selection of ideal strain for probiotic purposes might be difficult with regard to multiple criteria. So a series of established screening tests based on their importance were finalized for selection of the best isolate as a probiotic candidate. To exert beneficial effects adequate number of viable cells of probiotic agent should reach and colonize in the intestinal tract. Therefore, isolated *lactobacilli* were tested for aggregation, resistance to bile salt, acidic pH, various enzymatic tests, cell surface hydrophobicity test, ability to inhibit pathogens (antagonistic test), co-aggregation test and antibiotic sensitivity test.

4.4.1. Aggregation test

On the basis of the reports by Garriga *et al*. (1998) and Ehrmann *et al*. (2002), it is proposed that the aggregation test is appropriate for the first step of screening because it is a simple method applicable to a large number of test strains, and it shows clumping of strains together and also adhesion ability to the epithelial cells indirectly but in a strong way. Some of the *lactobacillus* isolates displayed high aggregation by settling down to the bottom of the tube upon keeping straight for 15 min. while others settled down completely within varying time period as given in the Table 8. Previous researches of Garriga *et al.,* 1998; Ehrmann *et al.,* 2002; Taheri *et al.,* 2009 clearly showed that the strains with high aggregation had better attachment to the epithelial cells. Taheri *et al.* (2009) reported that time needed for aggregation of *lactobacillus* as probiotic was generally between 10 and 120 min. which also favour our present finding. He reported that bacteria which show high aggregation or in other words low aggregation time also have high cell surface hydrophobicity and adhesion ability to the mucus.In this study isolates with short auto-aggregation time were selected as this clearly indicates the adhesion ability of isolates to the epithelium of the digestive tract.

4.4.2. Enzymatic activity test

All the *lactobacilli* isolates were screened for enzyme secreting ability like protease, phytase and lipase which facilitate digestion process.A probiotic that has the enzymatic activities can improve digestion especially in newly hatched chicks. The size of the halo zones surrounding the colonies/well inoculated with the culture of isolates or their cell free supernatant (CFS) gave an approximate indication of extracellular enzyme levels.

4.4.2.1. Protease activity test

Presence of halo zone surrounding the well punctured in 1% skim milk agar inoculated with lactobacillus isolate's CFS indicated production of protease enzyme by these organisms. Proteolytic activity of the isolates measured in terms of diameter of digestion zone varied significantly ($P<0.05$) among the isolates under study (Fig. 15). The proteolytic activity of these bacteria benefits the host by the liberation of the amino acids from feed or endogenous protein. These findings are in accordance with Taheri *et al.* (2009) who observed clear zone of digestion in MRS agar due to production of proteolytic enzymes.

4.4.2.2. Phytase activity test

The well diffusion assay in MRS agar with calcium phytate as substrate showed clear halo zones of varying diameters around the wells which showed that some of these isolates are capable of secreting phytase in a measurable amount which may increase digestion of these complex substrate and make phosphorous available to the birds while in some other isolates no phyatse activity could be detected Fig.16. Taheri *et al.* (2009) also observed varying phytase activity among *lactobacillus* isolates.

4.4.2.3. Lipase activity test

There was no halo zone around any of isolate inoculated well in the MRS agar medium which indicated there is no production of extracellular lipase enzyme by *lactobacillus* isolates (Fig. 17). which is in agreement with Taheri *et al.* (2009) and the report of Oterholm *et al.* (1968) who found that *Lactobacillus* do not have extracellular lipase production. There has been no previous report on the effect of probiotics on the intestinal lipolytic activity.

The enzymatic release assay (protease, phytase and lipase activities) is important to characterize the probiotic strain. Although bacterial isolates almost produced similar amounts of protease, they displayed different phytase activities in our study, but none of them exhibited lipase activity in well diffusion assay (Table 9).These results were similar to those reported by Kim *et al.* (2007) and Taheri *et al.* (2009).

4.4.3. Acid pH tolerance test

With regard to pH and retention time of feed in the gastrointestinal tract of the birds [crop (pH 4.5), 30min; proventriculus (pH 2.6), 15 min; gizzard (pH 4.4), 90

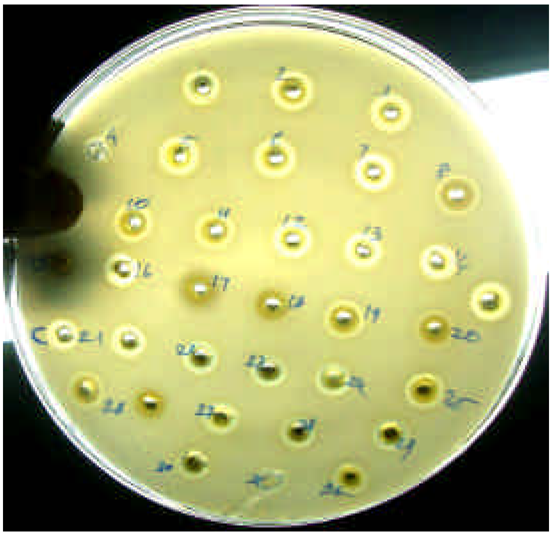

Fig. 15: Protease test (enzymatic test) for *Lactobacillus* isolates (CFS) in well diffusion assay showing zone of digestion

Fig. 16: Phytase test (enzymatic test) for *Lactobacillus* isolates (CFS) in well diffusion assay

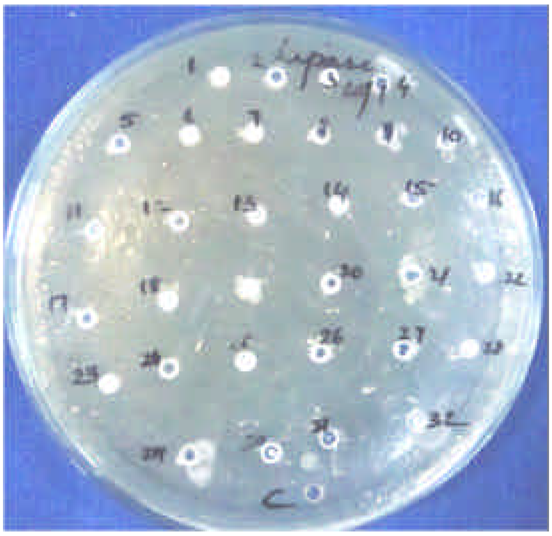

Fig. 17: Lipase test (enzymatic test) for *Lactobacillus* isolates in well diffusion assay

min; small intestine (pH 6.2), 90 min; and large intestine (pH 6.3), 15 min] (Church and Pond, 1974), it was thus preferred to examine the survivability of the strains close to the retention time of feed and pH in the gizzard. In the present study, viable colony of lactobacillus isolates on MRS agar after incubating at low pH indicated its tolerance / survivability at high acidic pH. Some of the lactobacillus isolates under study showed high resistance to even pH 2, almost all the isolates showed resistance to pH 3 after 90 min. of incubation (Table 9). Garriga *et al*. (1998) screened *Lactobacillus* isolates with regard to their tolerance to pH 3 and confirmed that that pH 3 did not decrease the number of isolates. The present results are in accordance to the reports by (Jin *et al*., 1998; Ehrmann *et al.*, 2002; Belkacem *et al.*, 2009 and Taheri *et al.*, 2009).

4.4.4. Bile salt tolerance test

The isolate's tolerance to bile salts is thought to be linked to their ability to produce beta hydrolase enzyme and thereby deconjugating bile acids (Tannock *et al.*, 1997). In the present work, the culture media containing 0.15, 0.3 and 1% (wt/vol) oxgall affected the growth of isolates in varying degree. Some showed reduced growth at higher concentration of bile salts while some showed apparent degree of survivability ie, tolerance level was found variable among all the test species of *Lactobacillus*, but 27 out of the 32 isolates tolerated 0.15% of bile salt concentration in the growth medium with significant difference among the isolates (Table 9). The results came in line with Havenaar *et al.* (1992), who observed resistance to bile salts is of great importance in survival and growth of bacteria in the intestinal tract and thus, is a prerequisite for probiotics. Taheri *et al.* (2009) reported 0.3 % and 1% bile salt inhibited growth of lactobacillus isolates but at 0.075 and 0.15% levels were tolerated by some isolates.

4.4.5. Cell surface hydrophobicity test

Hydrophobicity indicates the adhesion ability of *lactobacillus* to the mucus membrane and epithelium of intestine. Adhesion property of probiotic is very important to prevent the colonization of pathogenic bacteria (Martin-Pelaez 2009). In present study cell surface hydrophobicity was calculated as the percent decrease in the OD of *lactobacillus* bacterial suspension upon mixing with a hydrocarbon xylene. The hydrophobic nature of the outermost surface of the bacteria was examined photometrically. More than 90% of the isolates under study showed high hydrophobicity when treated with xylene with an average value 76.41±1.47 %, values for each isolate are given in (Table 9). The results were in agreement with Taheri *et al.* (2009) who could find higher surface hydrophobicity (85.21±7.27%). Values higher than 93% and between 66 to 93% were considered as strong hydrophobicity (Ehrmann *et al.,* 2002).A correlation of the hydrophobic nature of the outermost surface of bacteria has previously been implicated in attachment of bacteria to epithelial and intestinal mucosal cells (Rosenberg *et al.,* 1983; Slomiany and Slomiany, 1984; Handley *et al.,* 1987). Aggregation and cell surface hydrophobicity of the bacteria could be used for examination of adhesion ability to the mucus (Bujnakova *et al.,* 2004; Rahman *et al.,* and Taheri *et al.,* 2009), because there is a strong relationship among these characteristics especially between aggregation time and adhesion ability to the epithelium of the digestive tract (Garriga *et al.,* 1998). Our observations on aggregation and hydrophobicity were in agreement with those of Taheri *et al.* (2009) and Ehrmann *et al.* (2002).

4.4.6. Scoring of isolates

Decision for selection of ideal strain for probiotic purposes might be difficult regard to multiple criteria. Scoring of criteria are useful based on their importance for selection of the best bacteria as a probiotic. In the present study, a coefficient was defined for each criterion, named “Scoring Coefficient” that was multiplied to the result of particular test for each isolate. The summation of multiples makes final score for each isolate. A total of coefficient-1 was distributed among various probiotic quality assessment tests based on their importance. The criteria that involved for pH and bile salt tolerance were given high coefficient (coefficient=0.25 and 0.2, respectively) and enzymatic activity (coefficient=0.1 for each enzyme thus a total of 0.2, since no isolate showed lipase activity,it was not given with coefficient). Coefficient given for cell surface hydrophobicity was 0.1. Since the aggregation is a subjective test, scoring using coefficient was eliminated for this test .Those isolates scored high were then checked for their aggregation property,the isolate in which aggregation commenced within first 15 min. were selected for next phase of screening.

Five strains (LGFCP4, LGFCP6, LGFP16, LGFCM27 and LGFCM31) with good aggregation property, high pH and bile tolerance, high enzymatic activities, good

Table 9. Overall tests evaluations and scoring for different *Lactobacillus* isolates

Isolates	pHtolerance (pH-2)	Bile tolerance (0.15%)	Enzyme activity (dia of zone of digestion in mm)			Cell surface hydrophobicity	Score obtained
	% of survival		Protease	Phytase	Lipase	%	/isolate
LGFCP1	45.50±1.50^{k}	ND	15.45±.34^{abcdefg}	0.95±.05^{ij}	ND	70.0±1.0^{ghij}	**19.89**
LGFCP2	96.00±1.0^{abc}	ND	12.68±.44^{j}	0.95±.05^{ij}	ND	66.5±1.5^{ijk}	**32.013**
LGFCP3	97.00±1.0^{ab}	32.5±2.50^{de}	12.75±.58^{j}	0.95±.09^{ij}	ND	72.5±1.5^{efg}	**39.37**
LGFCP4	95.00±1.00	97.0±1.00^{a}	15.76±.66^{abcdef}	3.72±.07^{ab}	ND	87.5±1.5^{abc}	**53.848**
LGFCP5	99.00±1.00^{a}	27.5±2.50^{e}	16.32±.17^{abc}	4.00±.1^{a}	ND	91.5±2.5^{a}	**41.432**
LGFCP6	94±1.50^{abcd}	90.00±2.0^{cd}	15.59±.51^{abcdefg}	2.74±.06^{def}	ND	83.5±1.5^{cd}	**51.308**
LGFCP7	64.50±1.50^{i}	ND	15.95±.15^{abcd}	1.90±.69^{h}	ND	89.5±2.5^{ab}	**26.86**
LGFCP8	93.00±1.0^{bcde}	15.00±3.0^{f}	15.82±.28^{abcdef}	2.91±.03^{cde}	ND	82.0±2.0^{cd}	**36.323**
LGFP9	84.00±4.0^{g}	ND	15.83±.23^{abcde}	1.1±.1^{ij}	ND	55.0±1.0^{n}	**28.193**
LGFP10	90.00±2.00^{def}	91.00±2.0^{bcd}	15.63±.57^{abcdefg}	0.95±.05^{ij}	ND	77.5±2.5de	**49.808**
LGFP11	70.50±2.50^{h}	94.5±.50^{abc}	14.76±.31^{defghi}	2.09±.11^{gh}	ND	67.0±1.0^{hijk}	**44.91**
LGFP12	91.00±1.0c^{def}	93.0±1.0^{abcd}	14.82±.67^{cdefghi}	2.55±.54^{efg}	ND	60.5±1.5^{lm}	**49.137**
LGFP13	54.50±.50j	91.50±1.5^{bcd}	15.71±.53^{abcdef}	3.38±.18^{bc}	ND	56.5±1.5^{mn}	**39.484**
LGFP14	96.00±.00^{abc}	92.00±2.0^{abcd}	15.82±.69^{abcdef}	3.36±.08^{bc}	ND	75.0±2.0^{ef}	**51.818**
LGFP15	85.00±1.00^{g}	95.00±1.00^{abc}	16.32±.16^{abc}	.84±.06^{ij}	ND	93.5±.5^{a}	**51.316**
LGFP16	88.50±1.50^{efg}	94.50±.50^{abc}	15.48±.32^{abcdefg}	3.72±.08^{ab}	ND	86.5±.5^{abcd}	**51.595**
LGFI 17	86.50±1.50^{fg}	91.00±1.00^{bcd}	14.17±.03^{ghi}	3.87±.03^{ab}	ND	66.5±2.5^{ijk}	**47.779**
LGFI 18	90.00±2.0d^{ef}	94.50±1.50^{abc}	14.44±.46^{efghi}	3.05±.16^{cde}	ND	62.5±.5^{lm}	**49.399**
LGFI 19	34.50±.50^{l}	92.00±2.00^{abcd}	15.72±.30^{abcdef}	ND	ND	87.0±1.0^{abc}	**37.297**

contd...

LGFI 20	96.00 ± 1.0^{abc}	95.00 ± 1.00^{abc}	$13.85\pm.65^{hij}$	$3.28\pm.18^{bcd}$	ND	70.0 ± 2.0^{ghij}	**51.713**
LGFI 21	99.00 ± 1.0^{a}	91.00 ± 2.0^{bcd}	$14.96\pm.16^{bcdefgh}$	ND	ND	83.0 ± 2.0^{cd}	**52.74**
LGFI 22	97.00 ± 1.0^{ab}	ND	$14.85\pm.26^{cdefghi}$	$0.73\pm.06^{ij}$	ND	72.0 ± 2.0^{efgh}	**33.01**
LGFI23	90.00 ± 1.0^{def}	89.0 ± 1.0^{d}	$13.48\pm.62^{ij}$	$1.98\pm.1^{gh}$	ND	80.0 ± 2.0^{cde}	**49.84**
LGFI24	99.00 ± 1.0^{a}	90.5 ± 1.5^{cd}	$14.32\pm.21^{fghi}$	$2.24\pm.03^{fgh}$	ND	$83.5\pm.5^{cd}$	**52.85**
LGFCM25	88.50 ± 1.5^{efg}	86.50±1.5 d	$16.43\pm.36^{ab}$	ND	ND	$84.5\pm.5^{bcd}$	**49.58**
LGFCM26	99.00 ± 1.0^{a}	32.5±1.5 de	$15.64\pm.46^{abcdefg}$	ND	ND	64.0 ± 2.0^{kl}	**39.24**
LGFCM27	94 ± 1.5^{abcd}	90.0 ± 1.0^{cd}	$15.91\pm.27^{abcde}$	$1.3\pm.12^{i}$	ND	86.5 ± 1.5^{abcd}	**53.99**
LGFCM28	98.00 ± 2.0^{ab}	$95.50\pm.5^{abc}$	$15.09\pm.79^{abcdefgh}$	ND	ND	81.0 ± 2.0^{cde}	**53.20**
LGFCM29	94.50 ± 1.5^{abcd}	94.00 ± 1.0^{abc}	$15.95\pm.45^{abcd}$	$2.47\pm.3^{efgh}$	ND	75.0 ± 1.0^{ef}	**51.76**
LGFCM30	96.50 ± 1.5^{ab}	91.0 ± 2.0^{bcd}	$12.74\pm.32^{j}$	$0.57\pm.1^{jk}$	ND	92.0 ± 2.0^{a}	**52.85**
LGFCM31	90.00 ± 2.0^{def}	$94.5\pm.50^{abc}$	$16.30\pm.19^{abc}$	$0.96\pm.1^{ij}$	ND	86.0 ± 3.0^{abcd}	**51.72**
LGFCM32	95.50 ± 1.5^{abc}	88.50 ± 1.5^{d}	$16.49\pm.31^{a}$	$0.85\pm.0^{ij}$	ND	50.0 ± 2.0^{o}	**48.30**

Values are given in Mean±SE

Mean with different superscripts within column differ significantly ($p\leq0.05$)

cell surface hydrophobicity were selected for further screening tests like co aggregation and antagonistic tests.

Co-aggregation with *Salmonella* Typhimurium and *Salmonella*. Enteritidis and antagonistic test against *Salmonella* Typhimurium, *Salmonella* Enteritidis and *E. coli* were given a coefficient of 0.5 each. Based on these definitions, all the *lactobacillus* isolates were scored.

4.4.7. Co-aggregation test

Co-aggregation test has become the criterion for the selection of bacteria as probiotic since this factor shows the ability of *Lactobacilli* to interact with pathogenic bacteria. *Salmonella* and *E. coli* are common pathogenic bacteria that threaten the safety of GI tract in birds (Vicente *et al.,* 2008 and Higgins *et al.,* 2008). This test was conducted using selected five *Lactobacillus* isolate's culture mixed with *Salmonella* Typhimurium and *Salmonella* Enteritidis to study the co aggregation as indicated by% decrease in OD600 in the mixed sample after 4 hours of incubation at 37°C. Results of test are shown in (Table 10). The present finding showed a range of 3.01-13.76 % of co-aggregation between *Lactobacillus* isolates and *Salmonella* Typhimurium and 1.51-5.62% co-aggregation between *Lactobacillus* isolates + *Salmonella* Enetritidis which is in accordance with the findings of Taheri *et al.* (2009) who indicated that most isolated bacteria had poor co-aggregation of between 1 to 4.6 % and Hutari *et al.*, (2011) who got results between 1.0±2.0 to 13.8±2.1% while screening of *Lactobacillus* strains against *Salmonella,* both isolated from Malaysian free-range chicken intestine.

4.4.8. Antagonistic test

Antagonistic activity of *Lactobacillus* isolates was tested against *Salmonella* Typhimurium, *Salmonella* Enteritidis and *E.coli* and through well diffusion assay. Fig 18 a,b,c. shows the zone of decolonization around the wells punctured on nutrient agar inoculated with CFS of selected five isolates in our study indicating the inhibitory property of *Lactobacillus* isolates against *Salmonella* Typhimurium ,*Salmonella* Enteritidis and *E.coli* respectively. The mechanism of the antimicrobial action is due to the potential of *Lactobacillus* isolates to produce fatty acids, lactic acids, hydrogen peroxide, diacetyl, acetoin and the small, heat-stable inhibitory peptides called 'bacteriocins' (Soomro *et al.,* 2002; Simova *et al.,* 2009). The production of antimicrobial agents could be easily demonstrated *in vitro* by the well diffusion assay and the results obtained differed significantly among the isolates. The present findings were similar to those observed by Garriga *et al.* (1998), Miyamoto *et al.* (2000) and Reque *et al*. (2000). The values indicating the diameter of inhibition zone is given in the Table 11.

Based on the observations from *in–vitro* analysis of *lactobacillus* isolates for their probiotic potency, LGFCP4 (score =56.451) showed the highest score among other strains. LGFP16 (score= 54.045) got second place in score order.

4.4.9. Partial sequencing of 16S rDNA

The DNA of selected isolates (LGFCP4 and LGFP16) were isolated by conventional phenyl chloroform isoamyl alcohol method and 16S-23 S rRNA intergenic spacer region of each isolate was amplified using genus specific primers and amplicon size of approximately 250bp obtained to identify lactobacillus at genus level (Fig.19) (Dewhirst *et al.,* 1999, Tannock *et al.* 1999, Song *et al.,* 2000, Sui *et al.,* 2002, Blaiotto *et al.,* 2002) and PCR products were sequenced (partial sequencing of 16S rDNA) and its homology was analyzed through BLAST (NCBI). A 99% genetic identity for LGFCP4 and LGFP16 was found with *L.plantarum* and *L.reuteri* respectively. Neighbor-joining phylogenetic trees for both LGFCP4 and LGFP16 lab isolates from crop sample of guinea fowl and representative members of the genus *Lactobacillus* with bootstrap percentages (based on neighbor-joining analyses of 1000 resampled datasets) shown at nodes are given in Fig 20 and Fig 21.

4.4.10. Antibiotic sensitivity test

Selected lab isolated *Lactobacillus plantarum* (L.I.*L.p*) was tested against the sixteen most used antibiotics in veterinary medicine in India (Fig 22). Antibiotic susceptibility profile of L.I.*L.p* is shown in Table 12. The isolate was found resistant to Vancomycin, Streptomycin, Lincomycin, Ampicillin, Norfloxacin, Enrofloxacin, Sulphamethoxazole and Trimethoprim showed intermediate sensitivity to Kanamycin and Penicillin and showed high sensitivity to Tetracycline Clindamycin, Neomycin, Roxithromycin, Sulfafurazolidone and Nitrofurazone. Egervarn (2009) also observed that all *L.plantarum* strains were sensitive to clindamycin. Liasi *et al.* (2009) reported that *L.plantarum* were sensitive to penicillin and tetracycline and showed resistance to streptomycin, neomycin kanamycin, Sulphamethoxazole and trimethoprim. Liu *et al.* (2009) found that all *L.plantarum* strains studied possessed vanX gene, intrinsic

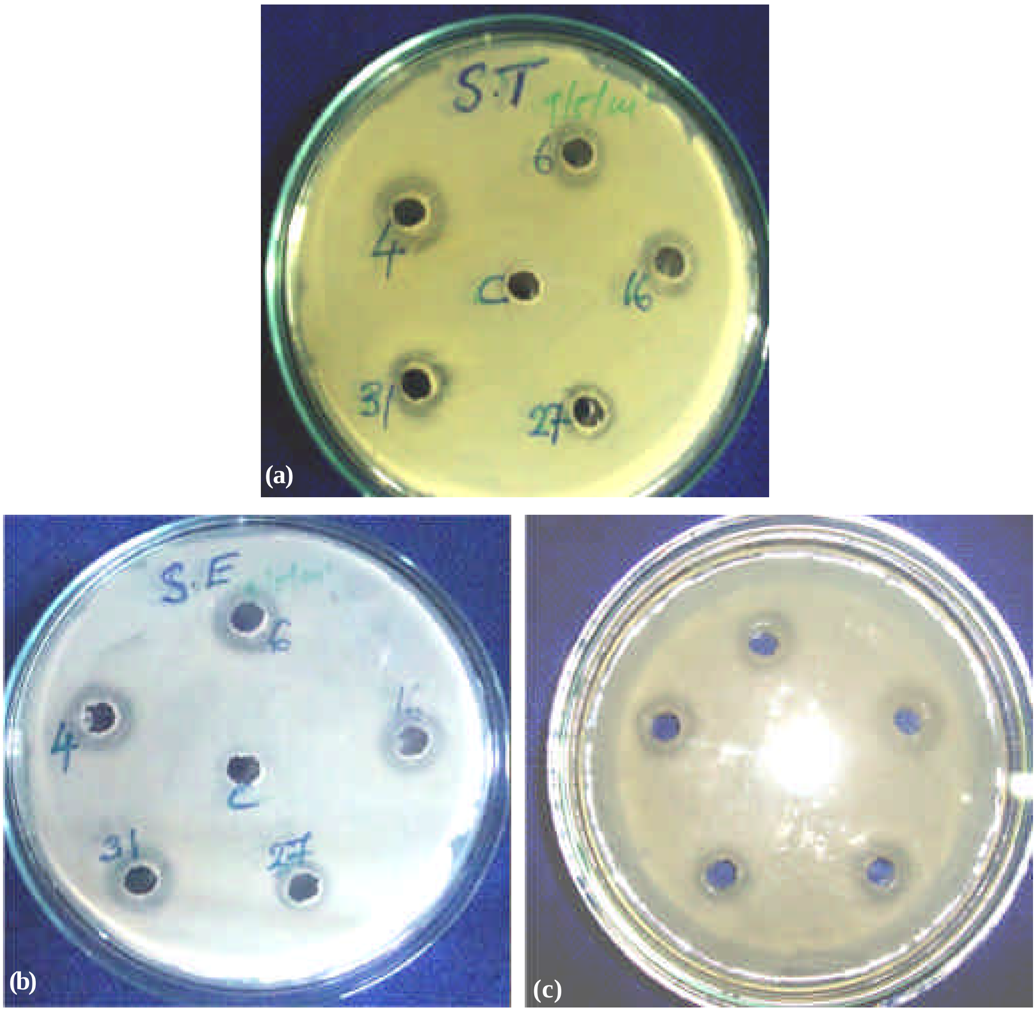

Fig. 18a,b,c: Antagonistic tests showing zone of inhibition by *Lactobacillus* isolates against S.Typhimurium, S.Enteritidis, and *E.coli* respectively

Fig. 19: Lane 1 and 3 shows PCR amplicon size of 250bp using primers targeted against 16S-23 S r RNA ISR in LGFCP4 and LGFP16 respectively. Lane 3 shows ladder of 100bp.

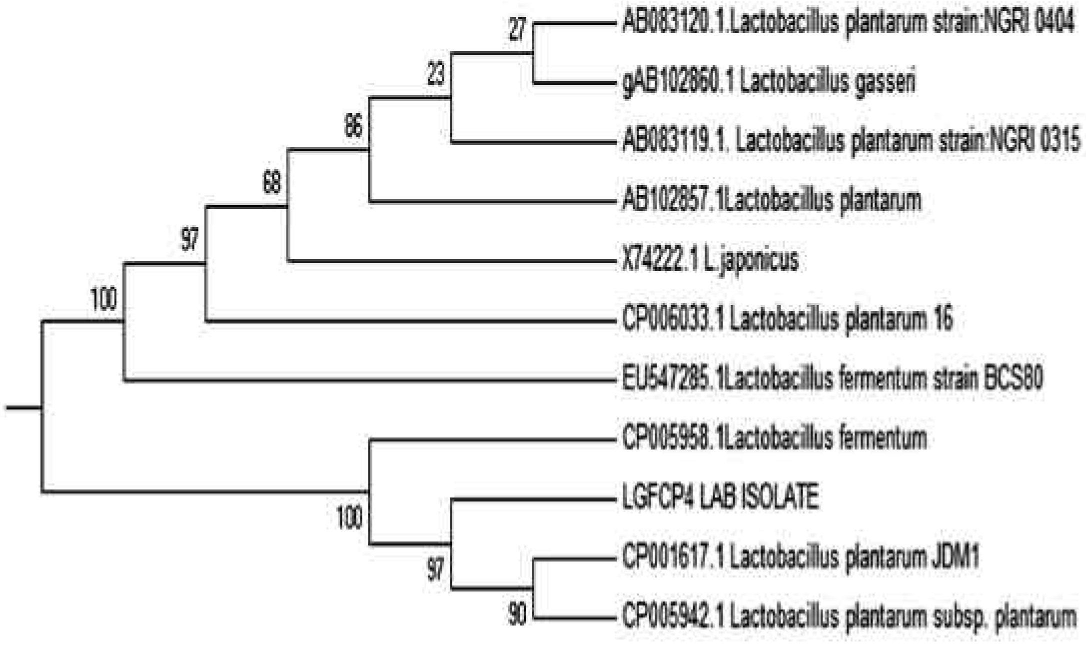

Fig. 20: Neighbor-joining phylogenetic tree, based on 16 S rRNA -23S rRNA intergenic spacer region gene sequences, showing the relationships between strain LGFCP4 lab isoalte from crop of Guinea fowl and representative members of the genus Lactobacillus. Bootstrap percentages (based on neighbor-joining analyses of 1000 resampled datasets) are shown at nodes.

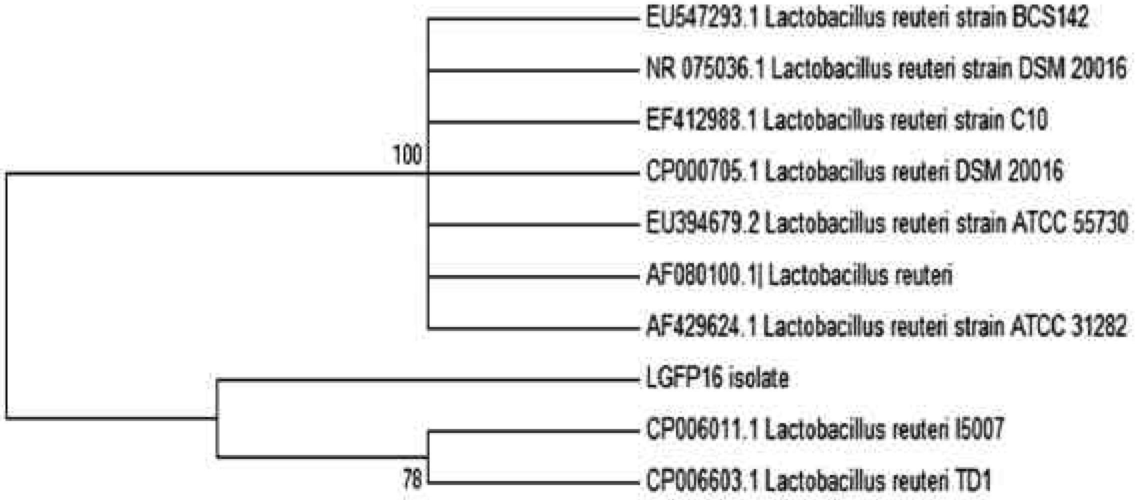

Fig . 21: Neighbor-joining phylogenetic tree, based on 16 S rRNA -23S rRNA intergenic spacer region gene sequences, showing the relationships between strain LGFP16 Lab isolate from proventriculus of Guinea fowl and representative members of the genus Lactobacillus. Bootstrap percentages (based on neighbor-joining analyses of 1000 resampled datasets) are shown at nodes.

Table 10. Co-aggregation test for *Lactobacillus* isolates using S. Typhimurium and S. Enteritidis

	***Lactobacillus* isolates (OD_{600} Values after 4 h incubation)**				
	LGFCP4	**LGFCP6**	**LGFP16**	**LGFCM27**	**LGFCM31**
LAB.	0.488	0.49	0.474	0.491	0.482
S.T	0.442	0.442	0.442	0.442	0.442
S.E.	0.438	0.438	0.438	0.438	0.438
LAB+S.T (4 hr.)	0.401	0.443	0.407	0.452	0.444
LAB+S.E (4 hr.)	0.437	0.442	0.433	0.458	0.451
% coaggreg (S.T)	13.76	4.94	11.14	3.01	3.89
% coaggreg. (S.E)	5.62	4.74	5.04	1.51	1.96
SCORE	**54.817**	**51.792**	**52.404**	**52.097**	**52.019**

OD of lactobacillus, S. Typhimurium, S. Enteritidis was adjusted to 0.5 then incubated for 4 hrs

Table 11. Antagonistic test against S. Typhimurium, S. Enteritidis and *E. coli*

Isolates	**Mean±SE of Diameter of zone of inhibition (mm)**			**SCORE**
	S. Typhimurium	**S. Enteritidis**	***E.coli***	
LGFCP4	12.82±.42^{a}	11.05±.51^{a}	8.81±.13^{a}	**56.45**
LGFCP6	11.23±.77^{a}	11.27±.40^{a}	8.05±.81^{a}	**53.31**
LGFP16	12.38±.73^{a}	11.61±.71^{a}	8.82±.38^{a}	**54.04**
LGFCM27	7.22±44^{b}	7.42±.44^{b}	5.19±.71^{b}	**53.08**
LGFCM31	11.96±.14^{a}	10.59±.45^{a}	7.93±.72^{a}	**53.54**

Mean with different superscripts within column differ significantly ($p \leq 0.05$)

Table 12. Antibiotic susceptibility profile of *L.plantarum* isolate

Antibiotic (dose/disc)	Susceptability
Vancomycin (30 mcg)	**R**
Kanamycin (30 mcg)	**I**
Neomycin (30 mcg)	**S**
Tetracycin (100 mcg)	**S**
Clindamycin (2 mcg)	**S**
Ampicillin (10 mcg)	**R**
Roxithromycin (30 mcg)	**S**
Nitrofurazone (10 mcg)	**S**
Streptomycin (10 mcg)	**R**
Norfloxacin (10 mcg)	**R**
Penicillin (10 units)	**I**
Sulfamethoxazole (300 mcg)	**R**
Sulfafurazole (100 mcg)	**S**
Lincomycin (10 mcg)	**I**
Enrofloxacin (10 mcg)	**R**
Trimethoprim (10 mcg)	**R**

R-Resistant, I-Intermediate susceptibility, S-Highly susceptable

Clinical Laboratory Standards Institute (2013)

Fig. 22: Antibiotic sensitivity test for *Lactobacillus plantarum*

Fig. 23: Fermented basal feed with addition of 15% broth culture(10^8 cfu/ml) of lab isolated *L.plantarum*

resistance gene supporting our work finding. Although it is clear that simultaneous application of susceptible probiotics with oral antibiotics is generally unreasonable, in the case of microbial infection it might be possible to use this probiotic candidate in combination with appropriate antibiotics by taking its antibiotic sensitivity profile into consideration. At the same time, antibiotic resistance development is a major issue among microflora, transfer of mobile genetic elements by resistant probiotic strains may possess a threat to livestock and human therapeutics.

4.5. Dose standardization of lab isolated *Lactobacillus plantarum*

After screening the *lactobacillus* isolate for its probiotic property bacterial enumeration was carried out for optimizing the bacterial count in culture. Broth culture was subjected for serial tenfold dilution and followed by plating to find out the bacterial count in order to standardize the culture for *lactobacillus* count. The average count of L.I.*L.p* was 1.05x10^{8}cfu/ml. Similar type of standardization of bacterial colony was done by Corrier *et al*. (1998). Similar type of dose standardization was done for *L.acidophilus* obtained from NCDC, Karnal for feeding trial.

4.6. Feed fermentation with isolated lactobacillus and ration formulation

As premix 20% of respective daily ration required for Guinea fowl and broilers was autoclaved and inoculated with 15% of *L.acidophilus* and lab isolated *L.plantarum* broth culture separately and incubated for 37°C for 24 hrs to facilitate fermentation (Fig 23).Owing to readily available source of energy in feed sample the count of *lactobacillus* in fermented feed increased to 1.91x10^{8}cfu/gm which was used in feeding trial (Savvidou *et al.,* 2009). During the entire experiment duration of 56 days in adult guinea fowl and 35 days in broilers, four treatment groups were provided with different dietary treatments (T1-basal diet (Control-1), T2-Antibiotic growth promoter BMD @ 20g /100kg feed (Control-2), T3-1x10^{8}cfu of *L.acidophilus*/gm fermented feed +MOS @ 1g /kg feed T4-1x10^{8} cfu of lab isolated *L.plantarum*/gm fermented feed+ MOS @ 1g /kg feed.There were 20 birds per each treatment group in Guinea fowl and 25 chicks in broilers. Discussions regarding the comparative effect of different dietary treatments used in feeding trial are mentioned below.

4.7. Growth Performance

The beneficial effect of any supplement is primarily judged through examining the response in terms of growth of birds for which it is intended to be offered as part of the diet. Accordingly, growth response of both adult Guinea fowl and broiler chicken to dietary supplement was an important part of the study, which includes the following aspects.

The response criteria of feeding trial like growth performance in guinea fowl and broilers and carcass traits in broiler viz., body weight, feed intake, feed conversion ratio, dressing%, carcass yield (evisceration yield), edible organ weights (giblet, liver, heart and gizzard%) and immune organ weights (thymus, bursa and spleen%) are studied.

4.7.1. Body weight

4.7.1.1. Guinea fowl

No significant difference was observed both in body weight and average body weight gain among the four dietary groups in adult guinea fowl at any period of time during the 56 days experiment (Table 13). However *L.acidophilus* and LI.*L.p* fed T3 (1702.73±19.9) and T4 (1691.15±9.66) groups showed better body weight over T2 (1678.17±11.28) and T1 (1677.9±15.54) group birds at the end of experimental period. Average body weight gain in T3 (270.0±5.78) and T4 (264.52±6.51) was also high when compared with T2 (260.21±4.07) and control group T1 (258.26±3.68) during the entire growth period.

4.7.1.2. Broiler-CARIBRO-Dhanraja

Average cumulative body weight at 5 week of age was observed as numerically higher but non-significant in T4 (1269.47±33.14), T2 (1265.00±24.69) and T3 (1262.06±34.64) group when compared with T1 (1208.12±27.01) i.e., unsupplemented group and average cumulative body weight gain was in a range from 1164.00±26.71 to 1225.31±32.92 with highest value in T4 and lowest in T1 group (Table 14).

4.7.2. Feed intake and FCR in broilers

Average feed intake per bird also differed among dietary groups. During 1-3 weeks period lowest feed intake per bird was observed in T2 (811.51), followed by T3 (811.59) and T4 (814.56) and highest feed intake was observed in T1 (833.82). Feed

Table 13. Growth performance in Guinea fowl fed on different dietary treatments

Parameters	**Treatment Groups (N-20) (Mean ± SE)**			**LI.*L.p***	**Significance**
	Control (T1)	**BMD (T2)**	***L.acidophilus* + MOS (T3)**	**+MOS (T4)**	**$p \leq 0.05$**
Body weight (gm/bird)					
Week 20	1415.90±39.88	1401.0±8.22	1426.87±11.32	1421.75±22.26	NS
Week 22	1420.0±76.12	1474.6±11.87	1512.40±22.23	1501.65±8.6	NS
Week 24	1510.95±79.00	1547.08±12.78	1561.50±21.78	1544.77±20.32	NS
Week 26	1553.04±68.331	1573.20±84.92	1634.36±9.79	1620.26±15.31	NS
Week 28	1677.9±15.54	1678.17±11.28	1702.73±19.9	1691.15±9.66	NS
Body weight gain (gm/bird)					
Week 20-22	71.4±1.36	72.7±1.98	80.60±1.98	78.65±1.58	NS
Week 22-24	65.10±1.85	68.47±2.75	71.41±2.15	69.10±1.92	NS
Week 24-26	60.4±1.47	62.78±1.20	65.34±1.72	64.92±2.83	NS
Week 26-28	54.73±1.77	56.60±1.59	58.68±1.42	56.70±0.76	NS
Week 20-28	258.26±3.68	260.21±4.07	270.0±5.78	264.52±6.51	NS

Table 14. Growth performance in broilers fed on different dietary treatments

Parameters	**Treatment Groups (N-25) (Mean ± SE)**			**LI.*L.p* +MOS (T4)**	**Significance $p \leq 0.05$**
	Control (T1)	**BMD (T2)**	***L.acidophilus* + MOS (T3)**		
Body weight (g/bird)					
Day 0	44.12±.54	43.64±.63	44.66±.52	44.16±.78	NS
Week 1	151.94±4.85	158.60±3.67	155.66±3.19	160.52±2.87	NS
Week 3	596.24±15.61	611.63±11.46	592.59±12.27	611.93±11.58	NS
Week 5	1208.12±27.01	1265.00±24.69	1262.06±34.64	1269.47±33.14	NS
Body weight gain (g/bird)					
0-7 Days	107.82±4.59	114.96±3.59	111.00±3.35	116.36±2.90	NS
1-3 Week	444.30±11.48	453.03±8.94	436.93±10.29	451.41±9.78	NS
3-5 Week	611.87±18.66	653.37±14.74	669.47±27.42	657.54±24.76	NS
0-5 Week	1164.00±26.71	1221.36±24.49	1217.40±34.70	1225.31±32.92	NS
Average feed intake (g/bird)					
0-7 Days	166.60	173.04	166.93	166.00	
1-3 Week	833.82	811.51	811.59	814.56	
3-5 Week	1209.12	1216.73	1233.25	1198.26	
0-5 Week	2203.94	2201.28	2211.77	2178.82	
FCR in various age group birds					
0-7 Days	1.54	1.49	1.49	1.44	
1-3 Week	1.87	1.79	1.85	1.81	
3-5 Week	1.97	1.86	1.84	1.82	
0-5 Week	1.9	1.8	1.82	1.78	

intake per bird during 3-5 weeks and cumulative feed intake were lowest for T4 with average values 1198.26 gm and 2178.82 gm, respectively. Feed conversion ratio (FCR) was lower for birds supplemented with LI.*L.p*+MOS during first week (1.44) over control birds (1.54). However, FCR at first week was similar for T2 and T3 (1.49), lower as compared to T1(1.54). During 1-3 week period, both BMD (1.79) and LI.*L.p*+MOS (1.81) supplemented T4 group showed lower FCR than *L.acidophilus*+ MOS supplemented T3 group (1.85) and T1 (1.87). During 3-5 week period, T4 group showed lowest FCR *ie,* 1.82. The cumulative FCR was lower for T2,T3 and T4 groups when compared to basal diet fed T1 group (Table 14). These findings are supported by Vandeplas *et al.* (2009) who reported that supplementation of *L.plantarum* increased daily body weight gain, decreased feed intake and improved FCR in broilers.

Since, probiotic microorganisms improve feed intake, digestion and feed conversion rate in broiler by production of extracellular digestive enzymes, FCR may be superior in probiotics fed group. Baghel and Singh, (2004) also found that the probiotics improved the production performance of poultry, established an environment to increase the digestibility of feeds and were a potential alternative to antibiotics in poultry diets.

4.8. Carcass analysis

4.8.1. Broiler

Pre slaughter weight of experimental broilers did not differ significantly ($P<0.05$) among different groups .There was no significant difference in dressing% (72.45±.47 to 74.03±.52), Carcass yield (67.18±.35 to 68.57±.44) relative weight of cut up parts to carcass weight between control group and supplemented groups. The means of relative weight of edible organs to the BW remained unaffected ($P>0.05$) by dietary supplementation and this result was supported by the findings of Ghavidel S.Z., (2011) and Salarmoini *et al.* (2011) that no significant effects were found on the relative weights of edible inner organs, thigh, liver, abdominal fat and carcass percentage in broilers fed with probiotic *lactobacillus*. The results of carcass analysis are shown in Table 15.

The LI.*L.p* + MOS supplemented group T4 had a greater ($P<0.05$) relative weight of immune organs to the BW compared with T3, T2 and T1 group. The weight of thymus was significantly higher in T4 group (0.32±0.04) over T1 (0.17±0.01), T2

(0.21±0.02) and T3 (0.20±0.01) groups. The weight of bursa was significantly ($P<0.05$) higher in T4 (0.33±0.05) over other three groups. However no significant difference was found ($P>0.05$) in relative weight of spleen between the groups (0.24±.02 to 0.26±0.01). The findings are supported by Patra, (2013) who observed significantly heavier thymus in domesticated japanese quails fed with isolated *Lactobacillus* sp. and no significant difference was found in spleen weight due to dietary treatments in quails.

4.9. Mortality

The data with respect to survivability of Guinea fowl and broiler chicken in different experimental groups have been set out in Table 16. It appeared that out of 80 adult Guinea fowl and 100 broiler chicks started in experiment, four adult Guinea fowl birds died during the trial, one bird from T1 and T2 during 22-24 week and one bird from T3 and T4 during 24-28 week period of experiment and no mortality was recorded in broilers. This shows that different dietary treatment had no particular effect on survivability of birds. The finding was supported by Bitterncourt *et al.* (2011.) who did not found any major difference in mortality among probiotic fed and control birds. Pelicano *et al.* (2004a) and Patra (2013) observed higher livability in birds fed on probiotics and prebiotics supplemented diet.

4.10. Influence of feed supplements on intestinal Salmonella and *E. coli* count

4.10.1. Guinea fowl

The least squares analysis of variance for $\log_{10}$ cfu/g of *Lactobacillus* spp., Salmonella spp. and *E. coli* in crop, ileum and caeca of in Guinea fowl fed with different treatments is presented in Table 19 and Fig. 26,27,28.

Enumeration of *Lactobacillus* spp., *Salmonella* spp. and *E.coli* was conducted at 56th day of feeding trial in Guinea fowl. Crop showed significantly ($P<0.05$) higher *lactobacillus* count in diets supplemented with *L.I.L.p* and *L.acidophilus* and mean $\log_{10}$ values for lactobacillus were 7.62±0.02 and 7.73±0.02 in T3 and T4, respectively. It is possible to assume that feeding lactobacillus increased their count significantly in intestine when compared to control birds. Whereas, lower $\log_{10}$ values (7.49±0.01 in crop and 7.41±0.03 in caeca) observed in T2 (antibiotic group) suggesting inhibitory activity of antibiotic on *lactobacillus.* In ileum similar trend was observed in T3 and

Table 15. Carcass traits in broilers fed on different dietary treatments

Parameters	Treatments (N-6) (Mean±SE)			LI.*L.p* +MOS (T4)	Significance
	Control (T1)	BMD (T2)	*L.acidophilus* + MOS (T3)		
Preslaughter weight	1260.40±23.67	1250.45±33.94	1269.41±40.76	1284.91±28.79	NS
Dressing%	72.45±.47	73.64±.67	73.31±.35	74.03±.52	NS
Evisceration%	67.18±.35	68.45±.55	68.03±.30	68.57±.44	NS
Neck%	4.19±.28	3.80±.24	4.06±.22	3.99±.18	NS
Wing %	11.50±.14	11.69±.23	11.74±.22	12.30±.19	NS
Breast %	25.36±.15	25.37±.41	25.59±.27	25.83±.25	NS
Back %	25.50±.09	25.98±.33	25.87±.28	25.51±.31	NS
Thigh %	16.23±.20	16.52±.20	16.47±.09	16.28±.18	NS
Drumstick%	15.99±.26	16.63±.23	16.26±.27	16.06±.09	NS
Giblet %	5.27±.14	5.19±.17	5.27±.17	5.46±.14	NS
Gizzard%	2.05±.08	2.08±.07	2.20±.11	2.14±.04	NS
Liver %	2.70±.05	2.56±.08	2.55±.08	2.62±.03	NS
Heart %	0.52±.01	0.54±.02	0.52±.02	0.53±.01	NS
Thymus %	0.17±.01^{b}	0.21±.02^{b}	0.20±.00^{b}	0.32±.04^{a}	$P<0.05$
Spleen %	0.24±.02	0.24±.01	0.26±.01	0.28±.01	NS
Bursa %	0.20±.01^{b}	0.21±.03^{b}	0.23±.02^{ab}	0.33±.05^{a}	$P<0.05$

Mean with different superscripts within row differ significantly ($p \leq 0.05$)

Table 16. Mortality in Guinea fowl and broilers fed on different dietary treatments

Treatments	**Guinea fowl**					**Broiler chicken**		
	No.of birds	**20-24 weeks**	**Mortality %**	**24-28 weeks**	**Mortality %**	**No.of birds**	**0-5 weeks**	**Mortality %**
Control (T1)	18	1	5.5	0	0	24	0	0
BMD (T2)	18	1	5.5	0	0	24	0	0
L.acidophilus +MOS (T3)	18	0	0	1	5.5	24	0	0
L.I.*L.p* +MOS (T4)	18	0	0	1	5.5	24	0	0

Table 19. Mean $\log_{10}$ value of *Lactobacillus* spp., Salmonella spp. and *E. coli* isolated from Guinea fowl intestine

Treatments	***Lactobacillus* spp.**			**Salmonella spp.**			***E. coli***		
	Log_{10} values of bacterial count (Mean ± SE)								
	Crop	**Ileum**	**Caeca**	**Crop**	**Ileum**	**Caeca**	**Crop**	**Ileum**	**Caeca**
Control (T1)	$7.52\pm.05^{bc}$	$7.48\pm.04^{b}$	$7.46\pm.04^{bc\ b}$	$5.79\pm.01^{c}$	$5.53\pm.02^{b}$	$5.96\pm.01^{c}$	$5.74\pm.01^{b}$	$5.63\pm.01^{c}$	$6.02\pm.07^{c}$
BMD (T2)	$7.49\pm.01^{c}$	$7.53\pm.01^{ab}$	$7.41\pm.03^{c}$	$5.39\pm.02^{b}$	$5.49\pm.01^{b}$	$5.49\pm.01^{a}$	$5.53\pm.02^{a}$	$5.49\pm.01^{a}$	$5.38\pm.01^{a}$
***L.acidophilus* +MOS (T3)**	$7.62\pm.02^{ab}$	$7.61\pm.02^{ab}$	$7.60\pm.05^{ab}$	$5.27\pm.02^{a}$	$5.54\pm.01^{b}$	$5.57\pm.01^{b}$	$5.56\pm.02^{a}$	$5.54\pm.01^{ab}$	$5.61\pm.05^{b}$
***LI.L.p*+MOS(T4)**	$7.73\pm.02^{a}$	$7.66\pm.06^{a}$	$7.70\pm.00^{a}$	$5.32\pm.02^{a}$	$5.27\pm.02^{a}$	$5.56\pm.01^{b}$	$5.51\pm.03^{a}$	$5.57\pm.02^{bc}$	$5.65\pm.02^{b}$

Mean with different superscripts within column differ significantly ($p \leq 0.05$)

Table 20. Mean log10 value of *Lactobacillus* spp., Salmonella spp. and *E. coli* isolated from Broiler chicken intestine.

Treatments	*Lactobacillus* spp.			Salmonella spp.			*E. coli*		
	Log$_{10}$ values of bacterial count (Mean ± SE)								
	Crop	**Ileum**	**Caeca**	**Crop**	**Ileum**	**Caeca**	**Crop**	**Ileum**	**Caeca**
Control (T1)	8.36±.01^{d}	8.78±.02^{b}	8.65±.02^{b}	7.35±.01^{c}	7.33±.01^{d}	7.2±.01^{b}	7.36±.01^{d}	7.18±.01^{c}	7.28±.01^{d}
BMD (T2)	8.64±.01^{c}	8.82±.02^{b}	8.61±.01^{b}	7.26±.02^{b}	7.21±.01^{c}	6.8±.06^{a}	7.16±.01^{b}	6.88±.01^{a}	6.76±.02^{a}
***L.acidophilus* +MOS (T3)**	9.05±.01^{b}	8.96±.01^{a}	8.86±.01^{b}	7.09±.01^{a}	6.72±.01^{a}	6.89±.01^{a}	7.22±.01^{c}	6.93±.01^{a}	7.12±.02^{c}
***LI.L.p*+MOS (T4)**	9.39±.01^{a}	8.98±.01^{a}	8.93±.01^{a}	7.06±.01^{a}	7.04±.03^{b}	6.98±.01^{a}	7.09±.01^{a}	7.07±.02^{b}	7.02±.02^{b}

Mean with different superscripts within column differ significantly ($p \leq 0.05$)

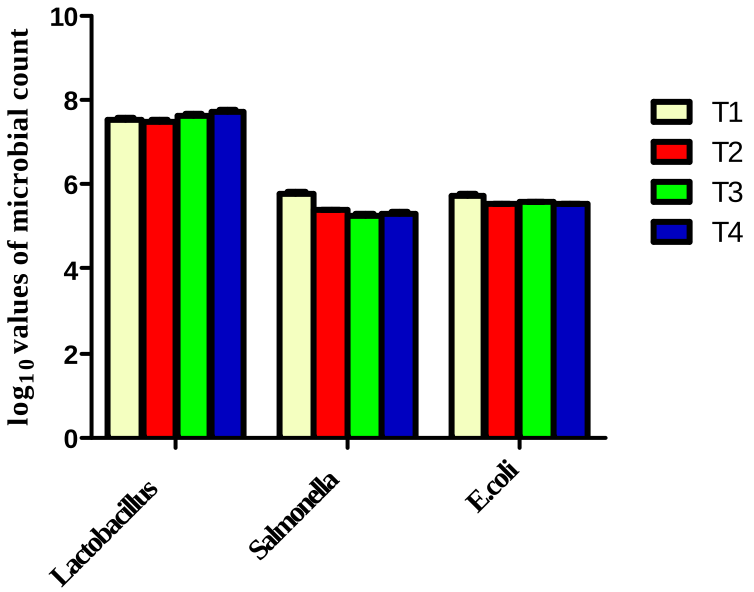

Fig. 26: Competitive exclusion analysis -Microbial count in crop of guinea fowl under different dietary treatments

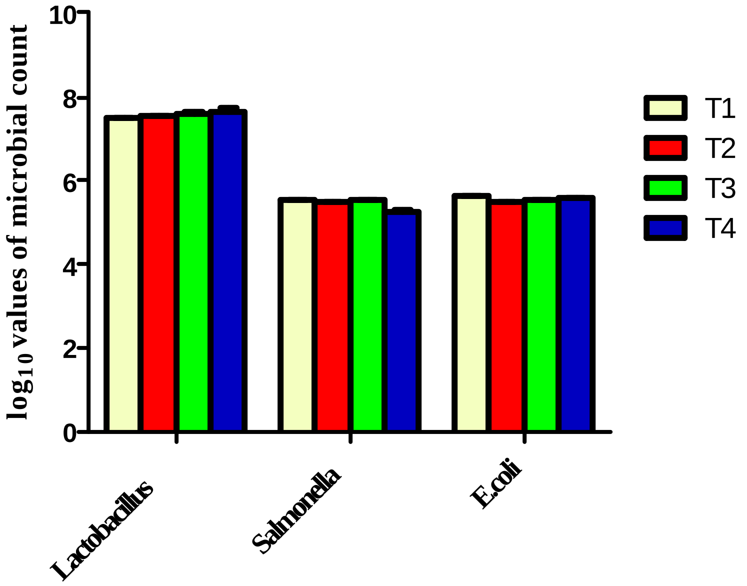

Fig. 27: Competitive exclusion analysis -Microbial count in ileum of guinea fowl under different dietary treatments

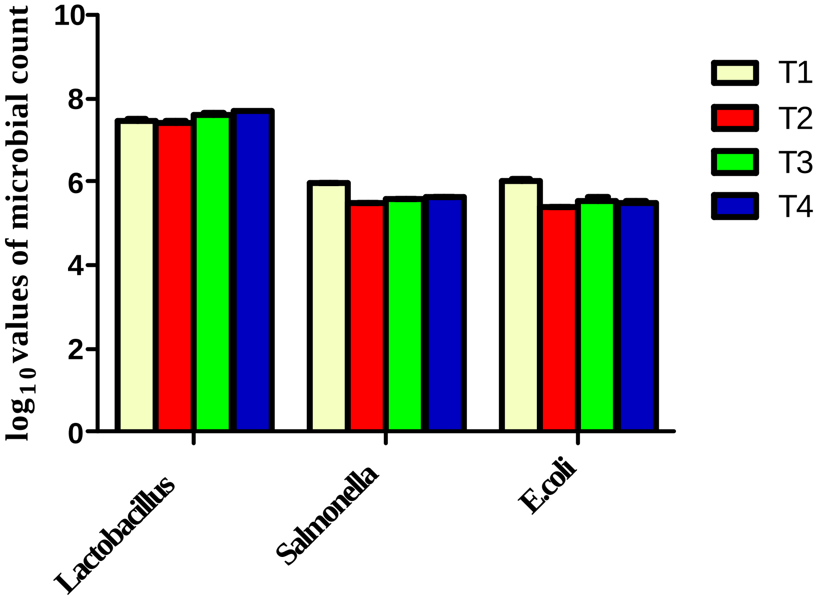

Fig. 28: Competitive exclusion analysis -Microbial count in caecum of guinea fowl under different dietary treatments

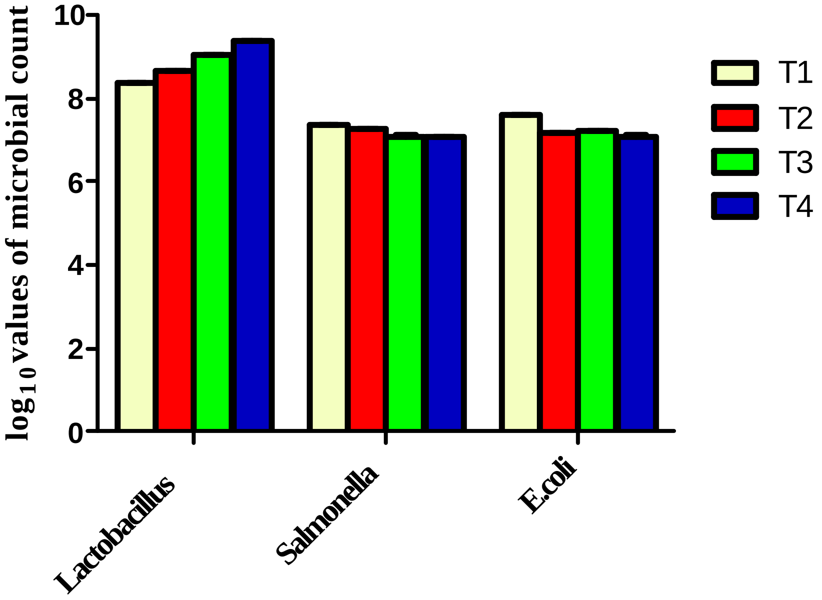

Fig. 29: Competitive exclusion analysis -Microbial count in crop of broilers under different dietary treatments

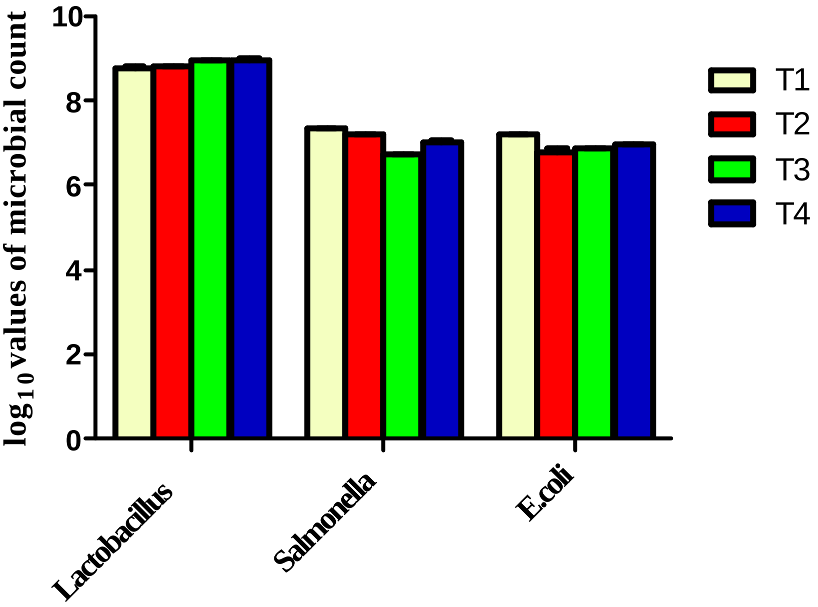

Fig. 30: Competitive exclusion analysis -Microbial count in ileum of broilers under different dietary treatments

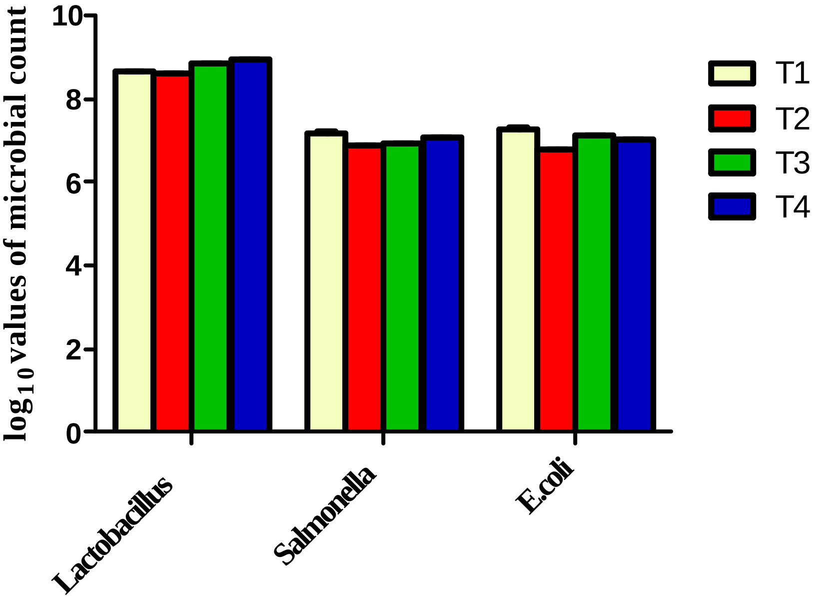

Fig. 31: Competitive exclusion analysis -Microbial count in caecum of broilers under different dietary treatments

T4 and lower count in T1 and T2 .Similar finding was observed in caeca with higher *lactobacillus* count in T4 (7.70±0.00).Whereas, lower in T2 and T1 group.

In terms of enumeration of *E. coli* was concerned in crop, significantly ($P<0.05$) lower count was observed in T2 (5.53 ±0.02) T3 (5.56 ±0.02) and T4 (5.51±0.03) suggesting the antagonistic activity of lab isolate and antibiotic could also reduce *E.coli* count. A higher count was observed in T1 (5.74±.01). In ileum and caecum T2 showed lower count (5.49±.01 and 5.38±.01, respectively) over T1, T3 and T4.

Salmonella count in crop was significantly low in T3 (5.27±.02) and T4 (5.32±.02) than T2 (5.39±0.02) and a highest count observed in T1 (5.79±0.01). In ileum lowest count observed in T4 over T1, T2 and T3. While in caecum antibiotic fed group T2 showed lowest salmonella count. There was no significant difference between T3 and T4 in caecal salmonella count.

4.10.2. Broiler

The least squares analysis of variance for $\log_{10}$ cfu/g of *Lactobacillus,* Salmonella and *E. coli* in crop, ileum and caeca of broilers fed with different dietary treatments is presented in Table 20 and Fig 29,30,31. Enumeration of *Lactobacillus* spp., *Salmonella* spp. and *E. coli* was conducted at 35th day of feeding trial. Crop showed significantly ($P<0.05$) higher *lactobacillus* count in diets supplemented with *L.I.L.p* and *L.acidophilus* and mean $\log_{10}$ values for lactobacillus were 9.05±0.01 and 9.39±0.01 in T3 and T4 respectively. A lower $\log_{10}$ values observed in T1 and T2 in all three parts of GIT. In ileum T3 and T4 group showed significantly higher lactobacillus count while T2 and T1 did not show significant difference. In caeca also T4 showed higher lactobacillus count than other three groups.

In terms of enumeration of *E. coli* count in crop, significantly ($P<0.05$) lower count was observed in T4 (7.09±0.01) suggesting the antagonistic activity of lab isolate and antibiotic also reduced *E. coli* count to a significant level. A higher count was observed in all parts of GIT of basal diet fed T1 group. In ileum T2 and T3 showed lower count (6.88±0.01 and 6.93±0.01, respectively).

Salmonella count in crop was significantly ($P<0.05$) low in T3 (7.09±.01) and T4 (7.06±.01) than T2 (7.26±.02) and a highest count observed in T1 (7.35±.01). In ileum lowest count observed in T3 (6.72±.01).While in caecum T2, T3 and T4 showed lowest salmonella count without a significant difference. In all three parts of GIT significantly ($P<0.05$) higher count of salmonella observed in T1 group.

From the study it can be concluded that feeding of lactobacillus isolate in Guinea fowl and Broiler chicken increased *Lactobacillus* count in different parts of GIT with concomitant decrease in pathogenic organism like *E.coli* and Salmonella. This suggests that lab isolate can be used as a potential probiotic candidate. The results were in accordance with Idoui *et al.* (2009), Patra (2013) and Ashraf *et al.* (2009) who reported reduction of *E. coli* count in intestine upon *lactobacillus* feeding.

4.11. Immune response

4.11.1.Humoral immune response

Data on the antibody levels detected in sera samples from Guinea fowl and broilers immunized with RD vaccine in terms of HI test was measured on 21st day post-vaccination have been summarized in Table 17 and Fig. 24. The $\log_2$ RD-HI antibody titre did not differed significantly ($P>0.05$) among different dietary groups even though highest titre in both Guinea fowl (6.12±.12) and broilers (6.62±.38) found in L.I.*L.p*. supplemented T4 group and lowest titre was found in T1 (5.50±.38) in broilers and T2 in guinea fowl (5.62±.32).

4.11.2.Cell mediated immunity (Lymphocyte proliferation assay)

The least squares analysis of variance for stimulation index in different treatments is depicted in Table 18 and Fig. 25. Blood was collected from the birds fed on different dietary groups on 21st day postimmunization and peripheral mononuclear cells PBMC were isolated and cultured in RPMI media for T-cell proliferation following mitogenic stimulation using PHA-P and determined the stimulation index (S.I.). There was significant difference among dietary groups. In Guinea fowl and broilers, T4 group birds showed highest S.I. ie, (1.57±0.04 and 1.81±0.03) followed by T3 (1.33±0.03 and 1.65±0.02) respectively. There was no significant variation in the value of S.I. in T2 (1.21±0.04 and 1.52±0.05) and T1 (1.20±0.06 and 1.53±0.06) groups. Stringfellow *et al.* (2011) and Mahmoud *et al.* (2013) observed the lymphocytes from vaccinated broilers treated with probiotic were found to have greater ($P\leq0.05$) cell proliferation when compared with the negative control group.

Table 17. Humoral Immune Response to ND in Guinea fowl and Broilers at 21st dpi

Treatments	Log$_2$ value of HI titre (Mean±SE)	
	Broiler chicken	**Guinea fowl**
Control (T1)	5.50±.38	6.00±.38
BMD (T2)	5.75±.31	5.62±.32
***L.acidophilus*+MOS (T3)**	5.75±.25	6.00±.19
***LI.L.p*+MOS (T4)**	6.12±.12	6.62±.38
Significance ($P \leq 0.05$)	NS	NS

Table 18. CMI -Lymphocyte Proliferation Assay: Stimulation index in Guinea fowl and Broilers in different dietary treatments

Treatments	Log$_2$ value of HI titre (Mean±SE)	
	Broiler chicken	**Guinea fowl**
Control (T1)	$1.53 \pm .06^{b}$	$1.20 \pm .06^{b}$
BMD (T2)	$1.52 \pm .05^{b}$	$1.21 \pm .04^{b}$
***L. acidophilus* +MOS (T3)**	$1.65 \pm .02^{ab}$	$1.33 \pm .03^{ab}$
LI.*L.p* + MOS (T4)	$1.81 \pm .03^{a}$	$1.57 \pm .04^{a}$
Significance	$P<0.05$	$P<0.05$

Mean with different superscripts within column differ significantly ($p \leq 0.05$)

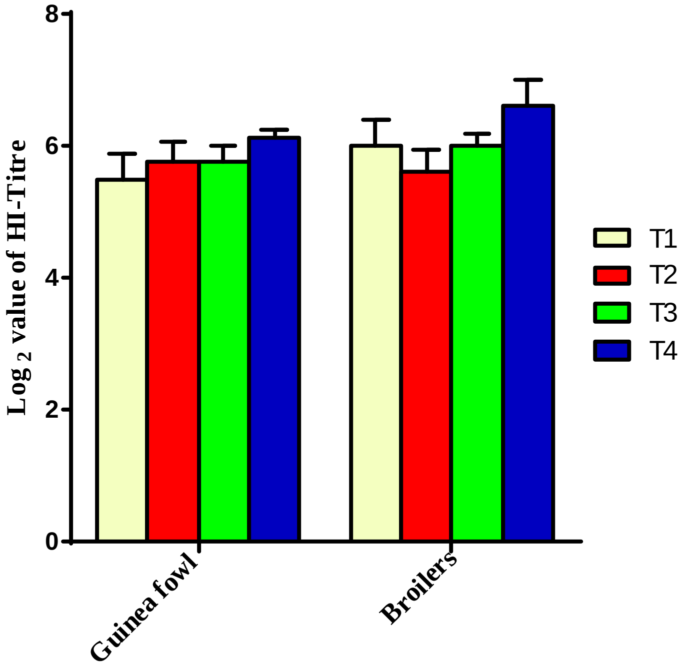

Fig. 24: Humoral immune response to ND vaccine in Guinea fowl and Broilers at 21st dpi

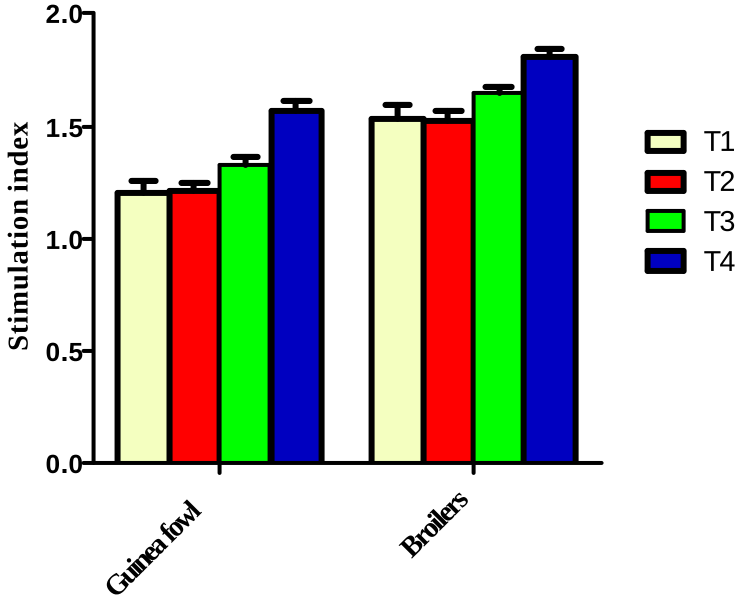

Fig. 25: CMI - Lymphocyte Proliferation assay in Guinea fowl and broilers

Table 21. Villous height, crypt depth (µm) and villus height/crypt depth ratio in duodenum and ileum of Guinea fowl and broilers on different dietary treatments

Treatments	**Duodenum**			**Ileum**		
	Villi height (µm)	**Crypt depth (µm)**	**Villi height/ crypt ratio**	**Villi height (µm)**	**Crypt depth (µm)**	**Villi height/ crypt ratio**
Broiler chicken (Mean±SE)						
Control (T1)	995.54±19.26^{b}	223.68±5.54^{b}	4.45±.14	952.15±25.14	172.40±3.84^{ab}	5.54±.13
BMD (T2)	925.55±18.22^{c}	212.51±4.14^{c}	4.36±.09	918.83±31.85	159.35±5.84^{b}	5.53±.20
***L.acidophilus* +MOS (T3)**	1047.63±18.44^{ab}	231.65±4.77^{a}	4.54±.15	967.63±19.62	174.53±4.41^{a}	5.59±.21
***LI.L.p*+MOS (T4)**	1060.56±20.44^{a}	235.57±4.23^{a}	4.50±.09	962.88±26.84	170.82±4.16^{ab}	5.64±.21
Guinea fowl (Mean±SE)						
Control (T1)	1330.20±20.48	313.80±10.4	4.25±.16	1168.20±22.5	263.48±7.16	4.45±.19
BMD (T2)	1280.72±22.96	300.2±6.28	4.27±.21	1120.92±31.7	257.32±5.62	4.35±.09
L.acidophilus +MOS (T3)	1334.44±24.09	317.00±11.31	4.23±.17	1158.4±46.98	263.92±7.48	4.39±.17
***LI.L.p*+MOS (T4)**	1334.56±21.01	311.4±10.06	4.24±.08	1206.56±32.9	264.70±4.04	4.3±.14

Mean with different superscripts within column differ significantly ($p \leq 0.05$)

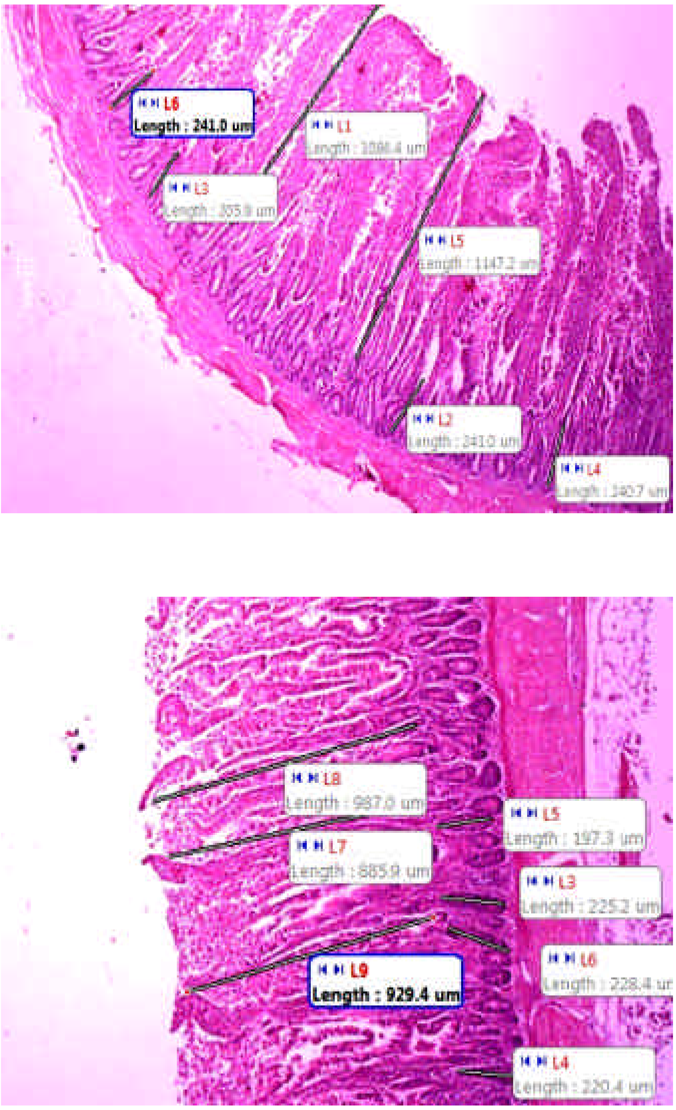

Fig. 32a,b: **Representative histomorphological photograph of duodenum and ileum showing villi length and crypt depth.**

4.12. Histomorphological examination

Histomorphological examination of duodenum, ileum of guinea fowl and broilers fed different dietary treatments was conducted at 56th day and 35th day of experiment respectively. In terms of histomorphology of intestine was concerned, duodenal villous height (VH) and crypt depth (CD) was significantly higher for T4 (VH-1060.56±20.44, CD-235.57±4.23) and T3 (VH-1047.63±18.44, CD-231.65±4.77) and lowest values obtained for antibiotic fed T2 (VH-925.55±18.22, CD-212.51±4.14) group. Whereas, ileal villous height didn't show any significant difference among treatment groups, ileal crypt depth was lowest for antibiotic supplemented T2 (159.35±5.84) group over the other three groups. Duodenal villous height:crypt depth ratio was highest for T4 (4.50±.09) and T3 (4.54±.15) over T2 (4.36±.09) and control birds T1 (4.45±.14). There was no significant difference in duodenal and ileal villous height,crypt depth,villous height crypt depth ratio among different dietary groups in guinea fowl eventhough high average duodenal villi height was observed in *lactobacillus* fed groups T3 (1334.44±24.09) and T4 (1334.56±21.01). Histomorphology results of small intestine observed in the present study are in agreement with the findings of Rahimi and Karimi (2005), Pelicano *et al*.(2005) that the supplementation of probiotic in broiler diets improved VH and CD in the small intestines of treated birds compared with the control birds. The increment in the size and number of intestinal glands and villi could result in greater enzyme production resulting in better digestion and absorption of nutrients (Mohan *et al.,* 1996). Parameters like duodenal, ileal villous height, cryptal depth and villous height cryptal depth ratio were measured and presented in Table 21 and Fig.32 a&b.

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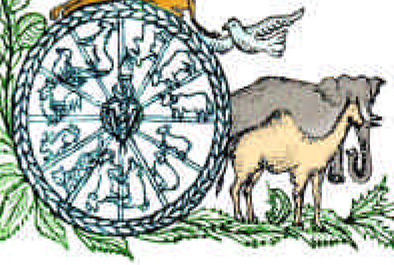
Summary and Conclusions



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SUMMARY AND CONCLUSIONS

The research work was undertaken to analyze the crop microflora of adult guinea fowl using Next Generation Sequencing (NGS) and bio-informatics tools which revealed 0.0026% archaea, 59.94% bacteria, 8.65% eukaryota and 31.17% viruses in the crop microbiome. A total of 43 species of *lactobacillus* genus were found in crop of guinea fowl with a number of strains under each species through metagenomic analysis. The study was also aimed to isolate and identify *lactobacillus sp.* from different segments of gastro intestinal tract of Guinea fowl followed by the screening of isolates for its probiotic potency tests. Initially a total of thirty two *lactobacillus* isolates eight isolates each from crop, proventriculus, ileum and caeca were characterized through morphological (colony morphology and Gram staining), Physiogical (growth at different concentrations of NaCl and at different temperatures) biochemical (catalase test and sugar fermentation tests) and molecular characterization (PCR amplification). For proving the efficacy of *Lactobacillus* isolates as probiotic candidate, screening was carried out through various *in vitro* probiotic quality assessment tests like aggregation test, resistance to bile salts and acidic conditions, enzymatic tests (protease, phytase and lipase test), cell surface hydrophobicity, co-aggregation, antagonistic test and antibiotic sensitivity test. Aggregation test displayed high aggregation in some of the isolates within first 15 min. upon keeping for 2 hrs. Most of the isolates showed resistance to pH 3 but significant difference was observed in their tolerance level to pH 2. A 1% concentration of oxgall inhibited the growth of all isolates while some isolates could survive on 0.3% bile salt addition in growth medium. MRS agar with 0.15% ox gall supported growth of all isolates with significant difference among them which indicates the *Lactobacillus* isolates can tolerate upto 0.15% bile salt. Distinct halo zone observed in enzymatic tests like protease, phytase tests indicates the digestive enzyme secreting ability of *Lactobacillus* isolates. All isolates showed almost similar protease activity whereas significant difference was found in phytase activity of the isolates. No isolate could secrete extracellular lipase enzyme.In cell surface hydrophobicity test gave an average value 76.41 ± 1.47 % ranging from 50.0±2% to 93.5±.5% indicating high hydrophobicity which shows the ability of isolates to adhere to mucus membrane.The isolates which showed lowest aggregation time also possessed high cell surface hydrophobicity which indicates a strong correlation between these characteristics. In co-aggregation tests a range of 3.01-13.76 % of co-aggregation between *Lactobacillus* isolates and S.Typhimurium and 1.51-5.62 % co-aggregation between *Lactobacillus* isolates and S. Enetritidis was found indicating adhesion ability of *Lactobacillus* isolates with pathogenic bacteria. Antagonistic activity of isolates, tested against *Salmonella Enteritidis* and *Salmonella Typhimurium* through well diffusion assay indicated the inhibitory property of *Lactobacillus* isolates against them.

After isolation, identification at genus level and probiotic potency assessment tests best two isolates LGFCP4 and LGF P16 were selected and partial 16 S rDNA sequencing was done to identify them at species level. LGFCP4 was identified with 99% genetic identity with *L. plantarum* and LGF P16 was identified with 99 % genetic identity with *L. reuteri* through BLAST analysis.The antibiotic sensitivity profile of *L. plantarum* with common antibiotics showed that isolate was resistant to Vancomycin, Streptomycin, Lincomycin, Ampicillin, Norfloxacin, Enrofloxacin, Sulphamethoxazole and Trimethoprim showed intermediate sensitivity to Kanamycin and Penicillin and showed high sensitivity to Tetracycline Clindamycin, Neomycin, Roxithromycin, Sulfafurazolidone and Nitrofurazone.

L. plantarum was selected for feeding trial in both Guinea fowl and broiler chicken after dose standardization of *L.plantarum* (10^8cfu/ml). Four treatment groups were provided with different dietary treatments (T1-basal diet (Control-1), T2-Antibiotic growth promoter BMD @ 20g /100kg feed (Control-2), T3 - 1x10^8cfu of *L. acidophilus*/gm fermented feed +MOS @1g /kg feed T4 - 1x10^8 cfu of lab isolated *L. plantarum*/gm fermented feed+ MOS @1g /kg feed. Parameters like body weight, FCR, immunity (cell mediated and humoral), microbial count and histomorphometry of duodenum and ileum were studied in Guinea fowl and broiler. In broiler chicken effect of dietary supplements on carcass traits were also studied.

There was no significant difference in average body weight and average body weight gain in both species, but feed intake and FCR was low in *L. plantarum* supplemented T4 group and *L. acidophilus* supplemented T3 group. Pre slaughter weight of experimental broilers did not differ significantly ($P<0.05$) among different groups. There was no significant differences in dressing % (72.45±.47 to 74.03±.52), carcass yield (67.18±.35 to 68.57±.44) relative weight of cut up parts to carcass weight and relative weight of edible organs to the BW between control group and supplemented groups.The means of relative weight of thymus and bursa of T4 group birds were higher than T1,T2 and T3 group.There was significant cell mediated immune response observed in Guinea fowl and broilers fed with *Lactobacillus plantarum* indicated by high stimulation index value in lymphocyte proliferation assay. Humoral immune response did not show any significant difference among groups. The data with respect to survivability of Guinea fowl and broilers appeared that different dietary treatment had no particular effect on survivability of birds.

So far as enumeration of *lactobacillus* is concerned, significantly higher *lactobacillus* count was observed in crop,ileum and caeca of both Guinea fowl and broilers in diets supplemented with isolated *L. plantarum* and *L. acidophilus* and mean $\log_{10}$ values for *lactobacillus* was lower in T-2 (antibiotic group) suggesting inhibitory activity of antibiotic on LAB and basal diet fed T1 group. Salmonella count was also found significantly lower in *L. plantarum* supplemented T4, T3 and T2 group in crop ,ileum and caeca over basal diet fed T1 group in both Guinea fowl and broilers.

In terms of enumeration of *E. coli* was concerned in crop, significantly lower count was observed in T4 suggesting the antagonistic activity of LAB isolate. But in ileum lowest *E.coli* count was found in T2 and T3 and caeca antibiotic supplemented T2 group showed lowest *E.coli* count followed by T3 and T4.

Histomorphology of intestine showed that duodenal villous height and crypt depth was significantly higher for T4 and T3 and lowest values obtained for antibiotic fed T2 group. Whereas, ileal villous height did not show any significant difference among treatment groups, ileal crypt depth was high for T4 and T3 and lowest for antibiotic supplemented group. Villous height:crypt depth ratio was highest for T4 and T3 over T2 and control birds. There was no significant difference in duodenal and ileal villous height,crypt depth, villous height crypt depth ratio among different dietary groups in guinea fowl eventhough high average duodenal villus height was observed in lactobacillus fed groups.

It is concluded that isolated *Lactobacillus plantarum* from guinea fowl GI tract has probiotic potential and has positive effect on growth, FCR, immunity, histomorphology of intestine and competitive exclusion property.Although the isolate was obtained from GIT of guinea fowl, present study shows that it can be utilized as a potential probiotic candidate in broilers also effectively.

CONCLUSION

Following conclusions are came out from the present study

1. Obtained a first report on crop microbiome of guinea fowl using metagenomic tools.
2. Confirmed the presence of *lactobacillus* in GIT of Guinea fowl through metagenomic study and conventional microbial cultural study.
3. Obtained clear picture on *Lactobacillus* abundance in crop microbiome of guinea fowl even upto strain level.
4. Screened thirty two *lactobacilli* isolates for their probiotic quality.
5. A score for ranking the isolates was developed on the basis of different assays.
6. The guinea fowl specific lab isolated *L. plantarum* found enhanced the immunity traits viz. Cell mediated immunity and weights of immune organs as well as exclusion of the pathogenic bacteria like *E. coli* and Salmonella in broilers and guinea fowl.
7. The guinea fowl specific lab isolated *L. plantarum* improved FCR in broilers.
8. The guinea fowl specific L.I.*L.p* may be used in commercial broiler production for enhancing immunity and improving growth. Further research is necessary to validate the findings in other poultry species also to develop a generalized probiotic combination specific for poultry nutrition.

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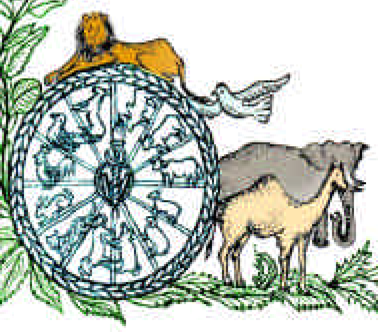
Mini Abstract



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MINI ABSTRACT

The proposed study was undertaken to analyze the gut microflora from crop of adult guinea fowl using Next Generation Sequencing (NGS) and bio-informatics tools which revealed 0.0026% Archaea, 59.94% Bacteria, 8.65% Eukaryota and 31.17% Viruses. Around 43 species of *Lactobacillus* were observed in crop microbiome of guinea fowl with a number of strains under each species through metagenomic analysis.The study was also aimed to isolate and characterize *lactobacillus* spp. from different segments of gastro intestinal tract of guinea fowl followed by the screening of isolates for their probiotic potency. A total of 32 *Lactobacillus* isolates were characterized morphologically (Gram staining), physiologically (growth at various temperatures and salt concentration) biochemically (catalase test and sugar fermentation pattern) and by molecular methods (PCR amplification).Screening was carried out for probiotic potency assessment of *lactobacillus* strains through different tests like aggregation test, resistance to bile salts and acidic conditions, enzymatic test (protease, phytase and lipase test), cell surface hydrophobicity, co-aggregation test, antagonistic test and antibiotic sensitivity test. The isolates showed significant difference among them for probiotic properties *in vitro*. Based on the results the isolates which scored highest points LGFCP4 and LGFP16 were sequenced and found 99 % genetic identity with *L. plantarum* and *L.reuteri* respectively through BLAST analysis. Then lab isolated *L. plantarum* was put into *in vivo* growth bioassay. 20% of daily ration for broilers and guinea fowl was fermented with 15% of broth culture having *L. plantarum* 10^8cfu/ml. Four treatment groups were provided with different dietary treatments (T1-basal diet, T2-Antibiotic growth promotant BMD @20g /100kg feed, T3-1x10^8cfu of *L.acidophilus*/gm fermented feed +MOS @1g /kg feed T4-1x10^8 cfu of lab isolated *L.plantarum*/gm fermented feed+ MOS @1g /kg feed). Through feeding trial, parameters like body weight, FCR, immunity (cell mediated and humoral), microbial count and histomorphometry of duodenum and ileum were studied in guinea fowl and broilers. In broilers effect of dietary supplements on carcass traits were also studied. Lab *isolated L. plantarum* at 10^8cfu dose was found effective in obtaining better FCR, improving cell mediated immunity, excluding salmonella and *E. coli* and increased duodenal villi height (VH) and crypt depth (CD) and VH:CD ratio in broilers which may in turn effect nutrient absorption in birds. Further research is necessary to validate the findings in other poultry species also to develop a generalized probiotic combination specific for poultry nutrition.

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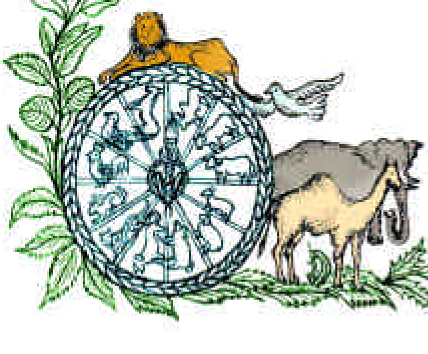

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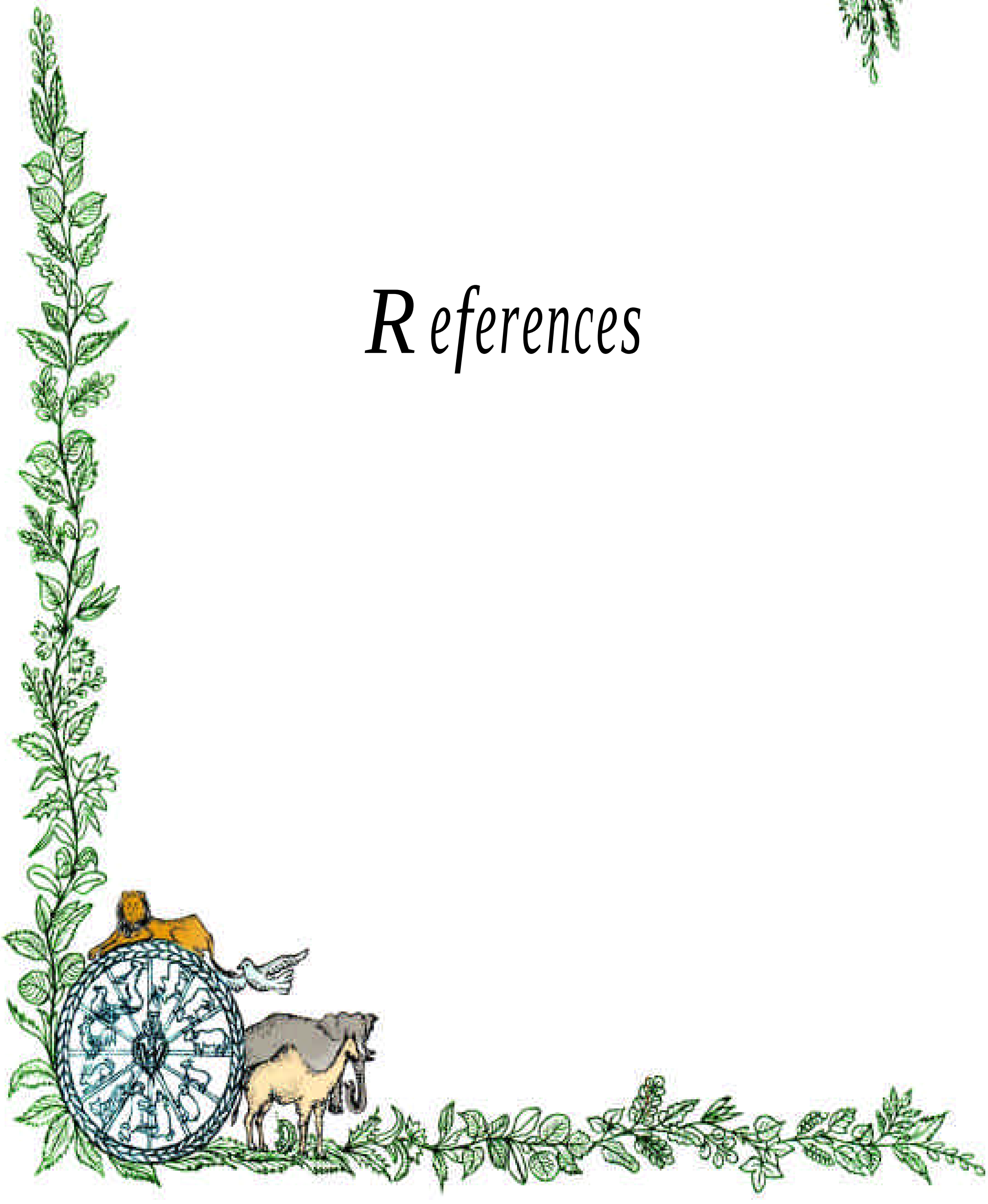

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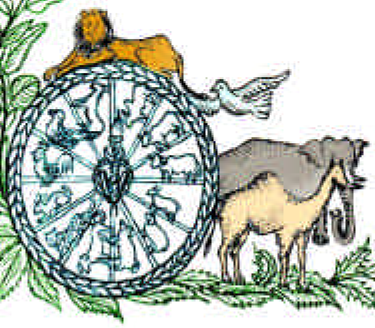
Appendix



APPENDIX-I

1. **Composition of PBS**

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44 g
KH2PO	4 0.2g
DW Up to 1000 ml	

2. **Composition of lysis buffer**

2M Tris HCl	0.5 ml
0.5 EDTA (pH-8.0)	0.2 ml
2M NaCl	2 ml
10 % SDS	5 ml
DW Up to 100 ml	

3. **Composition of TBE buffer (10x)**

TRIS base	108.0 g
Boric acid	55 g
Na EDTA $2H_2O$	8.3 g
DW Up to 1000ml	
pH 8.3	

4. **RPMI-1640 growth medium**

RPMI-1640 (Sigma ,USA)	10.40 g
HEPES (SRL, india)	5.92 g
β-mercaptoethanol (0.5M stock)	100 µl
$NaHCO_3$	2.20 g
L glutamate	2 mM
Fetal calf serum (Gibco)	100 ml
Streptomycin	100 µg/ml
Penicillin	100 IU/ml
Tripple DW made upto 1000ml	

The prepared medium was sterilized by membrane filtration (0.22µm), sterility tested for 48hrs and then stored at 4ºC for further use.

5. **MTT solution**

MTT (SRL,India) was dissolved in PBS at the rate of 5 mg/ml, membrane filtered and stored in dark at 4ºC for further use.

10% SDS (Sodium dodecyl sulphate)

SDS	10 g
DW upto	100 ml

Heat at 60ºC to dissolve and store at room temperature (adjust pH 7.2 using conc.HCl)

6. 0.5 M EDTA (pH 8.0)

EDTA	186.1 g
DW upto	1000 ml

Dissolve EDTA in about 800 ml of autoclaved distilled water by keeping it on magnetic stirrer for 1 hr then adjust the pH 8.0 by NaOH pellets before making into final volume. Autoclave and store at room temperature.

7. 3M Sodium acetate

Sodium acetate (anhydrous)	24.6 g

DW upto 100 ml

Adjust pH5.5 by using glacial acetic acid and sterilize by autoclaving.

8. 1M Tris HCl

Tris HCl	157.6 g

DW upto 1000ml

Adjut the pH 8.0 by NaOH pellets before making into final volume.Autoclave and store at room temperature.

9. Proteinase –K (20 mg/ml)

Proteinase –K	20 mg
Autoclaved DW	1 ml

Store at -20ºC.

10. 70% Alcohol

Ethanol	70 ml
DW	30 ml

11. 20,000 X ethidium bromide (10 mg/ml)

Ethidium bromide	10 mg
DW	1 ml

12. Taq DNA polymerase enzyme

Taq DNA polymerase	3U/µl

Store at -20ºC.

13.10X Taq buffer

Tris HCl (pH 8.8) 100mM

KCl 500m M

$MgCl_2$ 15mM

Triton X-100 1%

Store at -20ºC.

14. dNTPs solution in 100 µl

dATP 100 µM	(10 mM)
dCTP 100 µM	(10 mM)
dGTP 100 µM	(10 mM)
dTTP 100 µM	(10 mM)

Store at -20ºC

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