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Metagenomic analysis of intestinal microbiome of Indian male line broiler reared under different management systems

Arun TS, VK Saxena and Vineetha PG

Abstract
Effect of rearing systems on the gut micro biome of Indian male line broiler was investigated. Synthetic male line broiler developed at experimental broiler farm, CARI, Izatnagar was reared under intensive and extensive systems of rearing. Birds were humanely slaughtered at 5 weeks of age and whole intestinal contents were collected for NGS sequencing at Illumina (300 bp paired end) platform using primers targeting V_3, V_4, and V_4-V_6 hyper variable regions of 16srRNA. Microbial diversity was analysed using bioinformatics pipeline MG-RAST and Statistical differences for number of reads under various taxa revealed were analysed using Chi-square tests. The results revealed that gut micro biota differ significantly between rearing systems with total number of reads are quite high (334064) in chicks reared under extensive as compared to those reared under intensive system (198085).

Keywords: SML broiler, feed, metagenomics, illumina, intensive system, extensive system

1. Introduction
The gastrointestinal micro biota has one of the highest cell densities for any ecosystem and in poultry ranges from 10^7 to 10^{11} bacteria per gram of gut content (Apajalahti *et al*., 2004) [2]. The majority of these microbes are uncharacterized and represent an enormous unexplored reservoir of genetic and metabolic diversity. The gut micro-biota has an important role in poultry health and production, which generally affects the health of the host by influencing digestion and nutrient absorption, intestinal morphology, and defence of the host against infection (Mead 2000) [9].
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) [13]. Metagenomics can be used to address the challenge of studying prokaryotes in the environment that are, as yet, unculturable and which represent more than 99% of the organisms in some environments (Amann *et al*., 1995) [1]. Recent, advances in high throughput sequencing technologies have increased the number and size of metagenomic sequencing projects (Carola and Rolf, 2009) [4].
Bioinformatics tool like Meta Genomic Rapid Annotation using Subsystem Technology (MG-RAST) analysis provides a taxonomic classification and a new pipeline which computes results against many reference databases (GenBank, SEED, IMG, UniProt, KEGG and eggNOGs) (Meyer *et al*., 2008) [10].
Gut micro-biota is highly variable from individual to individual and also affected by several factors *viz.* environment, feed, genetic makeup of host etc. The modern day broiler strains have been produced through long term intense selection for production traits and are maintained under intensive system with feeding of compound feed. They are not adapted to extensive management system. Poltowicz and Doktor (2011) [11] reported that the housing system affected the rearing performance of broiler and lower body weight and higher mortality were reported in extensive system of rearing. Keeping this in view the present investigation was designed to find out the effect of rearing system on the gut microbial regime of Synthetic Male Line (SML) broiler which have been developed and maintained at Experimental Broiler Farm of the institute.

2. Materials and Methods
Synthetic male line (SML) broilers developed at experimental broiler farm, CARI, Izatnagar through long-term selection based on high body weight at 5-week of age, were used for the

investigation. Day-old chicks (10 chicks/ management system) were reared under intensive systems up to 5-weeks of age. Under intensive management chicks were provided controlled environment and ad-lib compound feed at Experimental Broiler Farm, CARI, Izatnagar. Under extensive system the chicks were maintained under rural conditions at farmer's door about 15 km away from institute. The chicks were housed in Kaccha houses made of locally available materials like asbestos sheet, card-board, mud etc. and fed on kitchen waste supplemented with broken grains and scavanging. The experiment was conducted during the month of December and February when ambient temperature ranged from 50.6 to 66.2°F and relative humidity 71-98%. Weekly body weights and mortality were recorded in both the system. Broiler reared under extensive system showed significantly lower weekly body weights (252g at 5th week) as compared to chicks under intensive system (995g at 5th week). The mortality was 45% in extensive system whereas 0% in intensive system.

Five chicks were humanely slaughtered at 5 weeks age and whole intestine contents were collected and pooled aseptically. The gut contents were outsourced to M/s Genotypic Pvt Ltd., Bangalore India for Next Generation Sequencing. V_3, V_4, and V_4-V_6 hyper variable regions of 16srRNA were amplified using region specific primers (Table-1) and NGS was done using Illumina 300bp paired end platform. The data generated were analysed using bio-informatics software, MG-RAST, a fully automated service for annotation of metagenomic data.

2.1 Statistical analysis

The statistical analysis was performed using Chi-square test-contingency table (2×2) for number of reads under various domains, phyla, class, order, and genus in the broiler chicks under both the rearing systems.

3. Results

3.1 Broiler under intensive system (IB)

During quality check by MG-RAST, 99.1% of total sequences (254866) passed quality checks based on length and number of ambiguous bases which represented the gut micro-flora using the V3,V4 and V4-V6 region of the bacterial 16S rRNA. Out of this 79.8% were predicted to be protein coding. Sequence similarity searches were computed against a protein database derived from M5NR database. Remaining 20.2% of the sequences hit ribosomal RNA. Source hit distribution against various 16S rRNA databases revealed that phylogenetic profiling of 51482 reads against Green genes database analysed 64.6% sequences. But only 0.03% of sequences could be analysed using SILVA LSU database. RDP could analyse 68.5% while SILVA SSU analysed 73.84% of the reads.

3.2 Broiler under extensive system (EB)

For broiler under extensive system, **9**9.1% (404298) of total reads passed quality check by MG-RAST which represent the gut micro-flora using the three regions (V3,V4 and V4-V6) of the bacterial 16S rRNA. Out of this 85.4% predicted to be protein coding. Sequence similarity searches were computed against a protein database derived from M5NR database. Remaining 14.6% of sequences hit against ribosomal RNA. Source hit distribution of these sequences 59027 sequences revealed that Green genes annotated 72.2%. Only 0.03% got annotated by SILVA LSU; RDP annotated 77.8% whereas 81% of total sequences got annotated using SILVA SSU database.

3.3 Gut microbial diversity of SML broiler under different systems of rearing

3.3.1 Phylogenetic profile at domain level

At domain level, gut micro-biota of broiler under both the systems of rearing were dominated by Bacteria which accounted for more than 90% of the gut micro biome (Fig.1). Viruses formed next major domain followed by Eukaryotes, Others and Archaea (Fig.1). Viruses include various types of Bacteriophages against different pathogens. Chi- square analysis revealed significant differences between the two management systems for number of reads (Table 1). Number reads for bacteria and viruses were higher in extensive system whereas those of eukaryotes and others were higher in intensive system; Archeal reads were almost similar in both the systems.

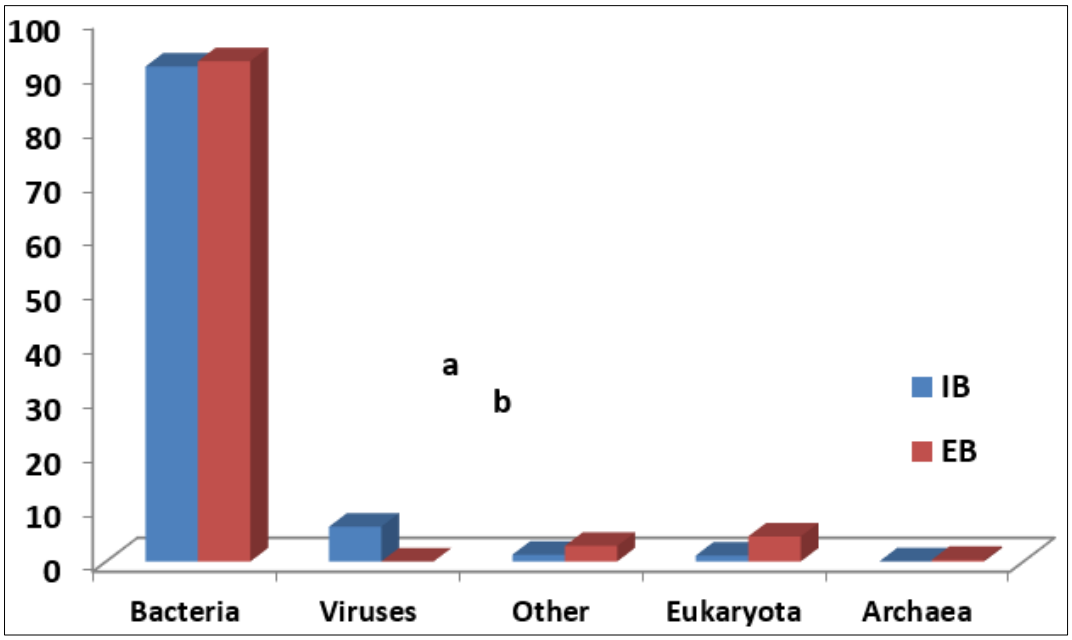

Fig 1: Domain level comparison of phylogenetic profile of SML under different systems of rearing

3.3.2 Phylogenetic Profile at phylum level

For broiler under intensive system, *Firmicutes* (76%) was dominant phylum followed by *Actinobacteria* (7%), *Proteobacteria* (4.6%) and *Bacteroidetes* (2.1%) (Fig. 2). Broiler under extensive system of rearing exhibited dominance of *Firmicutes* (71%), followed by *Bacteroidetes* (7.8%), *Proteobacteria* (7.2%) and *Actinobacteria* (2.7%). Firmicutes and Bacteroidetes are the beneficial bacterial phyla. In the case of broiler under intensive system, dominant eukaryotic phyla were *Arthropoda* (1.03%) and *Streptophyta* (0.05%) and dominant archaeal phylum was *Euryarchaeota* (0.013%) whereas, under extensive system of rearing *Arthropoda* (2.48%) and *Streptophyta* (0.3%) were the dominant eukaryotic phyla and dominant archaeal phylum was *Euryarchaeota* (0.008%). Number of reads at phylum level was found significantly different between the two management systems (Table 1). Extensive system broiler exhibited significantly higher reads number for Firmicutes, Proteobacteria, Bacteroidetes and Arthropods, whereas broiler under intensive system had higher reads of Actinobacteria and Cyanobacteria.

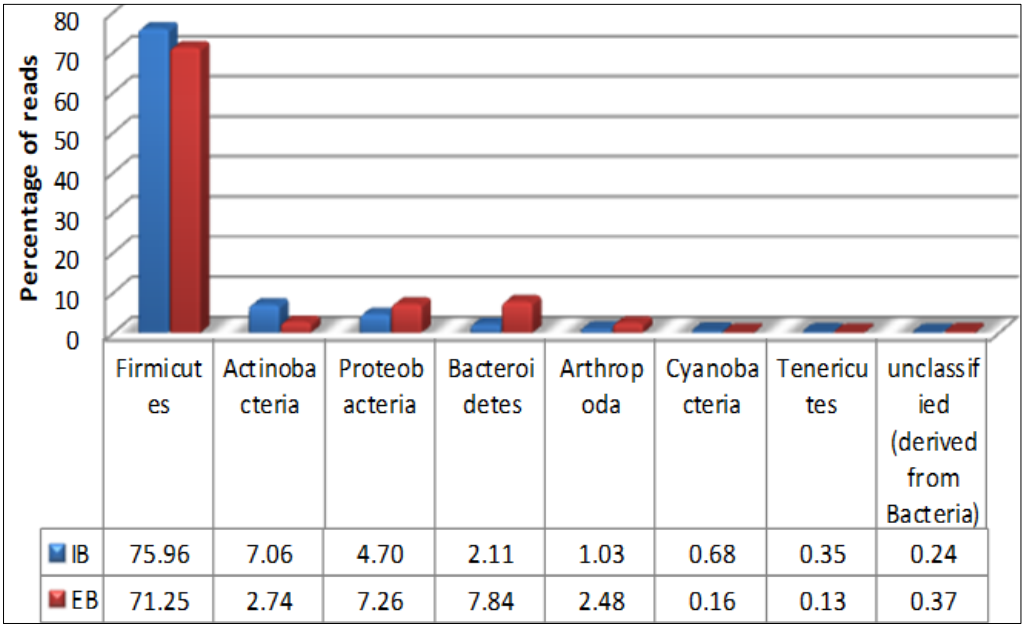

Fig 2: Phylum level comparison of phylogenetic profile of SML under different systems of rearing

3.3.3 Phylogenetic profile at class level

Phylogenetic profile at class level under intensive system exhibited that *Clostridia* (36.55%) and *Bacilli* (36.13%) were the pre-dominant classes followed by *Actinobacteria* (7%), *Negativicutes* (2.52%) and *Gammaproteobacteria* (2.34%). whereas, under extensive system the predominant classes were *Clostridia* (47.88%) and *Bacilli* (20.16%) followed by *Bacteroidia* (7.7%), *Deltaproteobacteria* (2.9%), and *Negativicutes* (2.8%) (Fig. 3). For broiler under intensive system of rearing *Insecta* (1.02%) was pre-dominant eukaryotes under intensive system whereas under extensive system, *Liliopsida* (0.2%) and *Arachnida* (0.05%) are dominant eukaryotes classes. Significantly higher read numbers for Clostridia, Negativicutes and Bacteroidia were found in broilers reared under extensive system whereas read numbers of Bacilli, Actinobacteria, and Gammaproteobacteria were significantly higher under intensive system (Table-1).

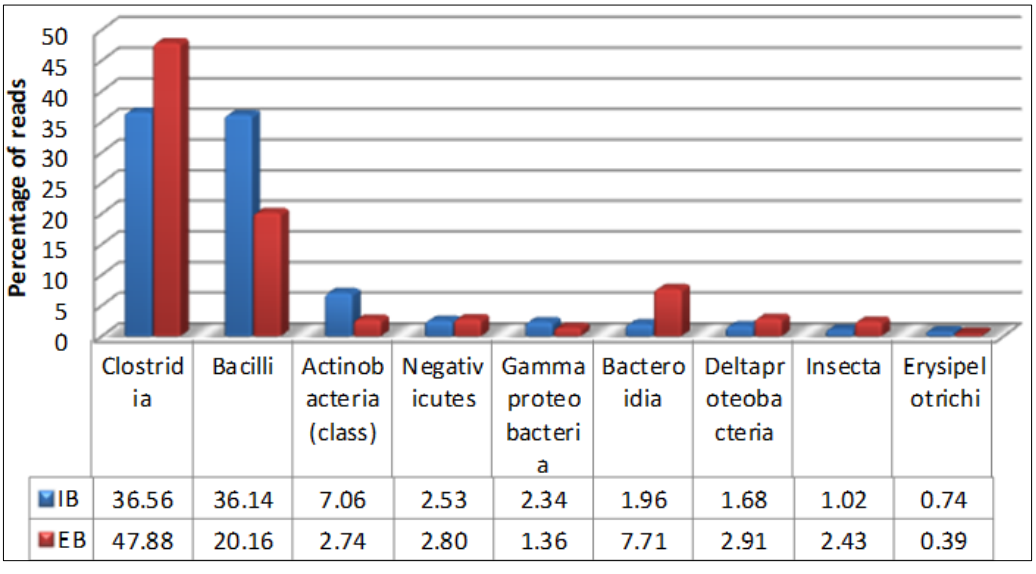

Fig 3: Class level comparison of phylogenetic profile of SML under different systems of rearing

3.3.4 Phylogenetic profile at order level
Clostridiales and *Lactobacillales* were dominant in broilers under both management systems, constituting 73% of total reads in intensive system and 68% in extensive system (Fig. 4). *Clostridiales* were higher in proportions compared to *Lactobacillales* in extensive system. Eukaryotes and arhaeal order constituted minor proportions under both the systems of management. Number of reads was significantly higher for *Clostridiales*, *Bacillales*, *Selenomonadales* and *Bacteroidales* under extensive system whereas *Lactobacillales* and *Coriobacteriales* had high read numbers in intensive system (Table-1).

3.3.5 Phylogenetic profile at family level
Streptococcaceae (14.14%), Lachnospiraceae (13.67%), Ruminococcaceae (11.17%), Lactobacillaceae (11%) and Enterococcaceae (8.19%) were the dominant families for broiler reared under intensive system. Whereas gut micro-flora of broiler reared under extensive system was dominated by Lactobacillaceae (12.9%), Ruminococcaceae (9.7%), Lachnospiraceae (8.22%), Clostridiaceae (5.64%), and Microviridae (4.6%) (Fig. 5). Eukaryotic and arhaeal families were in smaller proportions (< 0.1%) in broiler under intensive system. Rhinotermitidae (2.42%) had the highest reads among eukaryotic families in broiler under extensive system (Fig-5). Significantly high read numbers were recorded for Lachnospiraceae, Ruminococcaceae, Clostridiaceae and Veillonellaceae in extensive system whereas Streptococcaceae and, *Enterococcaceae* had higher read number in broilers under intensive system (Table-1).

3.3.6 Phylogenetic profile at genus level
Dominant genera for broiler reared under intensive system of rearing were *Lactococcus* (12.55%) and *Lactobacillus* (10.95%) followed by *Faecalibacterium* (9.5%), *Blautia* (8.37%), *Enterococcus* (7.96%) and *Clostridium* (4.08%). Gut micro flora of broiler reared under extensive system were dominated by genera such as Unclassified genus derived from *Clostridiales* (20.88%), *Lactobacillus* (12.8%) *Clostridium* (4.32%), *Ruminococcus* (3.82%), *Faecalibacterium* (3.17%), Blautia (3.12%) (Fig.-6). Eukaryotic and archaeal genera had small proportions (<0.1) in broiler reared under intensive system. Under extensive system of rearing *Coptotermes* (2.42%) was the predominant eukaryotic genus (Fig.-6). Genera *Lactobacillus*, *Clostridium* and *Eubacterium* had higher read number in broiler reared under extensive system whereas *Lactococcus*, *Faecalibacterium* and *Enterococcus* had higher reads intensive system (Table-1)

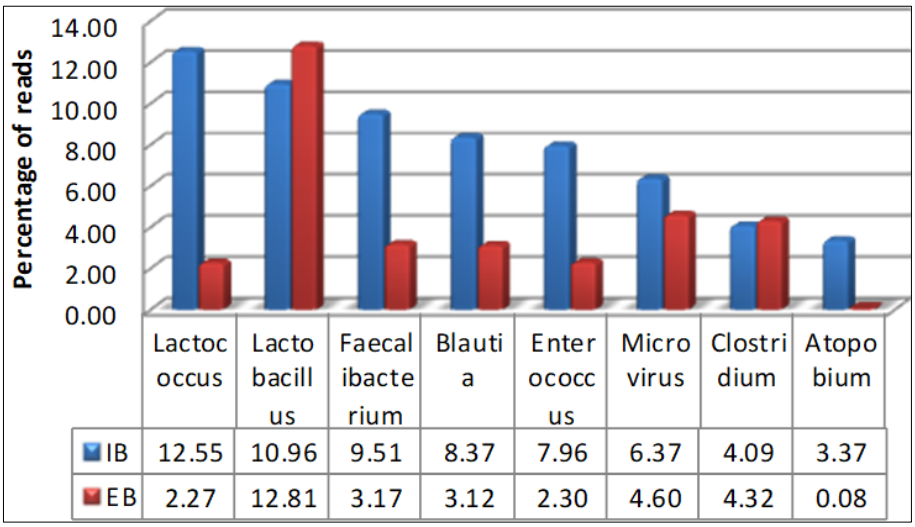

Fig 4: Genus level comparison of phylogenetic profile of SML under different systems of rearing

4. Discussion
The initial micro-biota to which chicks are exposed as well as the nutrient composition of diet affect their commensal gut micro biota, host gene expression, and immune system development (Yin *et al*., 2010) [18]. The SML broiler chicks reared under intensive system with standard feeding and husbandry practices. Whereas under extensive system, birds were fed on kitchen wastes supplemented with broken grains and reared under backyard condition with Kaccha house/night shelter. The lower growth rate and higher mortality shown by broiler under extensive system was due to the differences in feeds and rearing environment as compared to intensive system. The cold stress due to winter climate during experimental period would have also accentuated the situation. The birds under extensive management had to divert their energy resources more for maintenance of body temperature rather than for production/higher growth which would have led to reduced body weights in the chicks compared to those under intensive management. Malheiros *et al*. (2000) [7] also reported lower body weights under extensive management as well under cold stress.

The Taxonomic analysis at phylum level showed the dominance of *Firmicutes* followed by *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* in both the management systems which was in accordance with previous study conducted by Salanitro *et al*. (1974) [14] and Mead (1989) [8]. The ratio of *Firmicutes and Bacteroidetes* (F/B) has been found to be an indicator of growth in human. F/B ratio is significantly higher in obese individuals and is significantly reduced during weight loss (Ley *et al*., 2006) [6]. In the present study also the F/B ratio higher in broiler reared under intensive system (36.03) than those reared under extensive system (9.01).

Lactococcus garvieae, *Faecalibacterium prausnitzii*, *Enterobacteria* phage phiX174 sensu lato, *Lactobacillus sakei* and *Blautia* sp. Ser8 were the dominant bacterial strains for broiler under intensive system. *Lactococcus garvieae is a major fish pathogen* and its presence in chicken gut can be through fish meal ingredient for intensive feeding. *Blautia* sp. Ser8 is an anaerobic bacterium. Butyrate-producing bacterium

A2-232, *Lactobacillus sakei*, *Enterobacteria* phage phiX174 sensu lato, *Faecalibacterium prausnitzii* and *Coptotermes formosanus* were the dominant species for broiler under extensive system of management. *Lactobacillus sakei* is a probiotic strains which help in growth and immunity. Butyrate-producing bacterium A2-232 belongs to class clostridia and it helps in SCFA production. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium. Dominance of these species indicated that the adjustment of the host micro-biota to the changes in the feeding system since the broiler chick which were developed for intensive rearing had to utilize maximum nutrients out of the low energy kitchen waste and broken grains fed in extensive system. *Enterobacteria* phage phiX174 are phages against bacterial pathogens. Proietti *et al*., (2009) [12] also compared the intestinal micro flora in organic and conventional chickens and reported that the differences between the two groups, detected in bacterial count at the same age, are not sufficient to discriminate rearing systems. However, Valeria *et al*., (2007) [16] used T-RFLP to monitor the poultry gut micro-biota in response to dietary manipulations and found that diet-associated differences in gut microbial communities were detected within the ileum and cecum only. The dissimilarity in bacterial community composition between diets was 73 and 66% within the ileum and cecum, respectively. Litter management has also been reported to modulate the intestinal micro biome of broiler chickens which may have a profound effect on bird health and performance of broiler (Wei, *et al*., 2013) [17]. Singh *et al.* (2014) [15] reported that faecal metagenomes of high and low FCR birds revealed the sequences related to 33 genera in both groups but with significantly different proportion. Functional analysis revealed that genes for the metabolism of carbohydrates, amino acids and derivatives and protein metabolism were most abundant in SEED subsystem in both samples. Genes associated with stress, virulence, cell wall and cell capsule were also abundant. Indeed, genes associated with sulphur assimilation, flagellum and flagellar motility were over represented in low FCR birds. This difference seen in the composition of the micro-biota may be influenced by differences in the initial inoculums picked up from the egg and the early placement environment of the young chicks. Diversity and number of various taxa were more in broiler reared under extensive system.

Table 1: Chi square analysis for number of reads under different phylogenetic taxa

Management system	Phylogenetic Taxa					
	A. Domain					
	Bacteria	Viruses	Others	Eukaryote	Archaea	-
Intensive	180685^{a}	12713^{a}	2469^{a}	2188^{a}	30^{a}	-
Extensive	308013^{b}	15404^{b}	1017^{b}	9597^{b}	33^{a}	-
	B. Phylum					
Intensive	Firmicutes	Actinobacteria	Proteobacteria	Bacteroidetes	Arthropoda	Cyanobacteria
	150478^{a}	13993^{a}	9309^{a}	4176^{a}	2042^{a}	1349^{a}
Extensive	238015^{b}	9160^{b}	24261^{b}	26201^{b}	8295^{b}	528^{b}
	C. Classes					
Intensive	Clostridia	Bacilli	Actinobacteria	Negativicutes	Gammaproteobacteria	Bacteroidia
	72425^{a}	71593^{a}	13993^{a}	5006^{a}	4638^{a}	3878^{a}
Extensive	159983^{b}	67358^{b}	9160^{b}	9352^{b}	4547^{b}	25765^{b}
	D. Order					
Intensive	Clostridiales	Lactobacillales	Coriobacteriales	Bacillales	Selenomonadales	Bacteroidales
	71881^{a}	66566^{a}	11343^{a}	5028^{a}	5006^{a}	3878^{a}
Extensive	158411^{b}	60374^{b}	3601^{b}	6984^{b}	9352^{b}	25765^{b}
	E. Family					
Intensive	Streptococcaceae	Lachnospiraceae	Ruminococcaceae	Enterococcaceae	Clostridiaceae	Veillonellaceae
	28100^{a}	27168^{a}	22204^{a}	16275^{a}	10975^{a}	4868^{a}
Extensive	8781^{b}	27483^{b}	32400^{b}	7713^{b}	18854^{a}	9097^{b}
	F. Genus					
Intensive	*Lactococcus*	*Lactobacillus*	*Faecalibacterium*	*Enterococcus*	*Clostridium*	*Eubacterium*
	24969^{a}	21804^{a}	18913^{a}	15839^{a}	8132^{a}	3409^{a}
Extensive	7611^{b}	42869^{b}	10607^{b}	7688^{b}	14461^{b}	6311^{b}

Values having same superscripts in a column under each phylogenetic taxon between management system do not differ significantly (p<.01).

5. Conclusion

It may be concluded that Chi square analysis for number of reads under various taxa revealed that the gut micro flora of Synthetic Male Line broiler differ significantly under different rearing systems and environment influences development of gut microbes in broiler. The findings are indicative that shift in gut microbiome due to change in management and feeding system play vital role in sustenance of birds under adverse environment which tend to maximizing the feed utilization and immunity to birds in adverse conditions. Such bacterial communities which predominantly occupy gut microbiome of the high performing birds reared under adverse conditions may prove good probiotic under supporting environment for better growth and enhanced immunity to same genotype bird.

6. Acknowledgement

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