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EVALUATION OF MICROBIAL CONTAMINATION IN COMMERCIAL BROILER FEEDS AT KARACHI AND SURROUNDINGS

[a]Mehkar Hussain, [a]Iqra Ayaz, [b]Dildar Hussain Kalhoro, [c]Zulfiqar Ali Mirani, [a]Tanveer Abbas

[a] *Department of Microbiology, University of Karachi, Pakistan.*
[b] *Department of Veterinary Microbiology, FAH&VS, Sindh Agriculture University, Tandojam, Pakistan.*
[c] *Food and Marine Resource Research Centre, PCSIR Labs Complex, Karachi, Pakistan.*

A B S T R A C T

The poultry industry in Pakistan is a vital sector, accounting for 28% of the country's total meat production. Its growth rate is between 10% and 15% annually. However, if faulty components are used without proper sanitary precautions and feed is not thoroughly examined that can lead to the spread of diseases that harm both humans and animals. This research aims to identify and isolate disease-producing organisms in poultry feed to aid in diagnosis of microbial illnesses before treatment and control measures are implemented. During present study a total of hundred (n=100) Commercial broiler feed samples in triplets were analyzed according to Bacteriological Analytical Manual (BAM) (Drug Administration. Division of Microbiology. (1978). Bacteriological analytical manual (Vol. 1) by cultural techniques, microscopy, to detect the presence of Salmonella spp., *E. Coli*, coliforms, Y&M, APC, and fecal coliforms. Then each isolate was identified by biochemical testing that included Indole, Methyl Red, Voges-Proskauer test, Citrate Utilization, Motility, Gas and Lactose tests. Out of 100 samples, 97 had an APC count, 68 showed growth for Y&M, 27 had coliforms and fecal coliforms, 13 had *E. coli*, 12 had positive growth for Salmonella spp., statistics valid in the form of overall percentage (%) detected microbial contaminants in feed samples, showed that out of all 100 samples, 40% had an aerobic plate count, 28% had mold and yeast, 11% had coliforms, and 11% had fecal coliforms. In entire samples, *E. coli and Salmonella* was detected only 5% each. It is concluded from present study that pathogens like *Salmonella*, *E. coli*, yeast, and molds were isolated and identified from commercial broiler feeds may be a source of salmonellosis, colibacillosis, mycotoxicosis hence causing financial losses because of low FCR, treatment cost and drop in production and significant burden on public health because of use of antibiotics for infected birds treatment may be a source of Anti-Microbial Resistance. Continuous Scientific research, aseptic handling, buying top quality ingredients, hygienic production of feeds, proper silos and storage facilities with regular check and balance for ingredients through R&D labs should be ensured for minimizing contaminants.

Key Words: AMR; Broiler; BAM; *E. coli; FCR; Salmonella*

Corresponding Author: Mehkar Hussain
Email: organizer.pvdf@gmail.com

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INTRODUCTION

Globally, the broiler meat, is one of the widely admired meat, with a projected faster demand and consumption (Chai, 2017). As the growth trajectory of the poultry sector has shown, modern feed is vital for animals with modern genetics. Appropriate disease outbreaks surveillance and management may protect animals' health and economy. Disease-free zones with a complementing vaccination regime can be pillars of livestock-based exports *'The State of Pakistan's Agriculture 2023, PBC'.* Several factors contribute to the growth of the poultry industry are

expanding global population, increasing purchasing power for developing economies, urbanization and industrialization, advancements in feed development and transfer, short production cycles, enhancements in poultry breeding, conservation, and improved processing technologies (Dei, 2017).

Application of precision nutrition enables feed mills to provide various diets for blending on poultry farms in real-time, for achieving nutrient requirements for sustainable poultry production (You, 2024). Poultry feeds consist of cereals like maize, rice or barley, wheat, oilseeds cake meal, and protein-rich animal byproducts like fish meal, meat and bone meal, slaughterhouse offal, and feather meals (Sibanda, 2023). For offering great positive effects, including promoting growth, boosting immunity, nutritional supplements and additives are becoming essential in today's poultry industry and in health-care scenario too (Gilbert, 2017). Enzymes, Antibiotics, pro-biotic, and organic acids are only a few of the feed additives benefiting broiler feed (Elgeddawy et. al., 2020) are included into broiler and other animals diet for growth stimulation by perhaps better intake (Mahrose et. al., 2019).However, a significant portion of these feed additives and ingredients serve as a key source of microbial intervention, posing potential risks to public health (Tarabees, 2017). Microbial contamination in agricultural products can lead to food safety and animal health issues, with consumers exposed to microbiological dangers due to stressed conditions and poor animal care. Proper cleanliness is crucial to prevent foodborne illnesses and economic burden (Douphrate, 2021) (Cruciani, 2022) (Gomes, 2023) (IPBES, 2020).

Microbial organisms specially *Salmonella*, *E.coli*, yeast and molds could be found in poultry feeds and can cause severe infections in poultry, and their transmission to humans could also be possible, so, this may show concern about great financial losses to the poultry industry and a major burden of human-health (Ngai, 2021). Because of increased economic losses to poultry industry, this research proposal is designed, to diagnose the microbial diseases of poultry, by isolating and identifying disease-producing organisms, before treatment and control measures are instituted. The following goals have been established in order to accomplish the goal: separate yeast and mold from feed samples, calculate the bacterial load using the total aerobic count, All samples, were subjected to coliform and fecal coliform isolation, and each isolate was identified by biochemical testing.

METHODOLOGY

Microbiological Media & Reagents

The research involved, culture media and reagents such as Lauryl Tryptone Broth (LTB), RV/Selenite Cystine Broth, Lactose Broth, Butterfield's phosphate buffer, Xylose Lysine, Desoxycholate Agar, Dichloran Rose-Bengal Chloramphenicol (DRBC) Medium, Nutrient Agar, Nutrient Broth, PCA (plate count agar), TSB (Tryptic Soy Broth), EMB (eosin methylene blue) and MacConkey.

Sample size and Collection

A convenient sampling method was adopted. Owners of different poultry farms and commercial feed market places at Karachi and surroundings were contacted. From selected areas i.e. Gadap, Dabeji, Northern bypass, Korangi, Hub-Chowki, Winder and Nooriabad, a total 100 broiler feed samples (each of 250g) of different feed brands used by these farmers were collected aseptically and shifted to Food and Marine Resource and research Centre, PCSIR Labs Complex, Karachi, Pakistan in the year 2023.

Figure 1. Poultry feed samples collected from commercial farms / mills at Karachi.

Laboratory Procedures

The study followed the guidelines mentioned in the Bacteriological Analytical Manual (BAM) (Drug Admin. Div. of Microbio. (1978). B.A.M. (Vol. 1). Association of Official Analytical Chemists).

Sample Preparation

50g of each feed sample were introduced into peptone water. Intensive shaking of the flasks ensured thorough amalgamation of the samples by peptone water. Subsequently, the flasks were spared for 10-15min to facilitate sample settling. Once settled, immediate potentization, conducted by pouring 10ml from first 10^{-1} dilution, in vessel having 90-ml of Butter-field's phosphate buffer. This serial dilution process was replicated till 10^{-3}.

Analysis of Mold and Yeast Count

For the assessment of yeast and mold, 0.1 ml, from the instance dilutions (1:10, 1:100, and 1:1000) withdrawn, and applied to apparent solidified DRBC agar-dishes. Each dilution was allocated to three separate plates. Post-application, in the absence of light the agar plates underwent incubation for five Days at 25°C.

Microscopic Examination of Fungi Isolates

Microscopic examination of fungi isolates involved extracting a small portion of suspected fungi from media and depositing them on a slide in stain (Danbappa et al., 2018).

Analysis of Bacteria

For bacteriological analysis, both general and selective media were used. General media included Nutrient broth (NB), Nutrient agar (NA), and PCA, while selective media included RBS Broth, TSB, EMB, and MacConkey (Khan et al., 2015).

Aerobic Plate Count

From each serially diluted tube upto 10^{-3}, cultured the sample on nutrient agar plates, by spread plate technique while for PCA, pour plate technique is used, by transferring 1 ml from each serially diluted sample, into the media. Kept the tubes in incubator, at 37C for overnight. On the next day, counted the colonies on each plate, that, were considered as Aerobic Plate count (APC). Tests repeated and then, all the feed samples were processed in same manner.

Detection of *Salmonella*

Pre-enrichment

Salmonella identification involved pre-enrichment in Tryptic Soy Broth, centrifugation at 12000 rpm for 2 minutes, and incubation for 24 hours.

Direct culture of samples on selective media

Bacterial cultures, from various feed samples were directly placed on selective media. One gram of each sample was gotten, and individually poured into a glass-beaker containing 9ml of PBS. After thorough mixing, the contents were inoculated onto selective media, for the identifying and isolating *Salmonella and E. coli spp*. Incubation of these media occurred at 37 °C one-night.

Sub-culturing on Selective Media

For *Salmonella* identification, selective enrichment involved inoculating 0.1ml of culture into 10 ml of RV/Selenite broth, poured 1ml of culture into 10ml Tetrathionate broth. RV/Selenite broth tubes were incubated at 42 °C for 24 hours, while Tetrathionate broth tubes were incubated at 43 °C for 24 hours. Following incubation, a loop full of culture from both broths was cultured on two sets of Bismuth Sulphite Agar, Hektoen Enteric Agar, Xylose Lysine, and Desoxycholate Agar. Now at 35^{o}C Incubation was again done for 24 hours, and cultural characteristics were examined upon completion.

E. *Coli* Identification

For *E. coli* identification, colonies from Nutrient Agar plates were transferred to Eosin-Methylene Blue Agar (EMB) by wire loop. Then, the plates be incubated overnight at 37°C. Simultaneously, content from nutrient broths of diverse feed samples was inoculated into selective media and kept in an incubator at 37 °C overnight.

Identification by Colonial Morphology, Gram Staining for Bacterial Isolates and Biochemical Tests

After a 24-hour incubation under varied conditions, the form, size, colour, roughness, edge and elevation of surface, and opacity of the colonies were meticulously observed and documented (Khan et al., 2015). Smears of each isolate from the plates, be prepared by extracting a minute section of microbial growth. Subsequent staining and counter-staining steps were performed to examine cell morphology, arrangement, capsules presence, and reactions of stains (Onajobi et. al., 2023).The suspected isolates were examined, by Biochemical tests and included Indole, MR-VP, Citrate Utilization, Motility Test, Gas and Lactose tests (Bhutia et al., 2021).

Statistical Analysis

The descriptive statistical analysis was carried out utilizing**,** all 100 samples that were processed in triplets for getting more authentic results and to detect any variation for proper statistical analysis. Using SPSS 27.0 version software, the data for various biochemical parameters, was analyzed statistically by the frequency of occurrence and distribution procedure in MS Excel 2016, for the processing and tabulation of obtained data. Utilizing standard errors of means, the outcomes were interpreted (Sharma et al., 2015), which are mentioned in results section.

RESULTS

A total 100 samples (n=100) of broiler feed, evaluated against APC, Y&M, coliforms, fecal coliforms, *E.coli* and *Salmonella* spp. Out of those samples, APC was observed in 97 samples and no count was shown in remaining samples. For Y&M, growth was observed in 68 samples while there was the lack of growth in 32 samples. Coliforms and fecal coliforms were detected in 27 samples out of 100 samples. In case of *E.coli*, it was detected in 13 samples out of 100 samples. And 12 samples showed growth, while remaining samples were negative for *Salmonella spp.* (Figure 2).

Analysis of Mold and Yeast Count

The analysis conducted on samples revealed concerning levels of molds and yeast contamination and suggests potential health hazards and fungal quality issues. All the samples were diluted and transferred on the surface of the solidified Dichloran Rose- Bengal Chloramphenicol (DRBC) agar plates and were incubated for 5 days at 25 ^{o}C, in the dark. After the 5 days of incubation, growth on plates was recorded and their colony characteristics were observed properly. To confirm the growth, microscopy was performed.

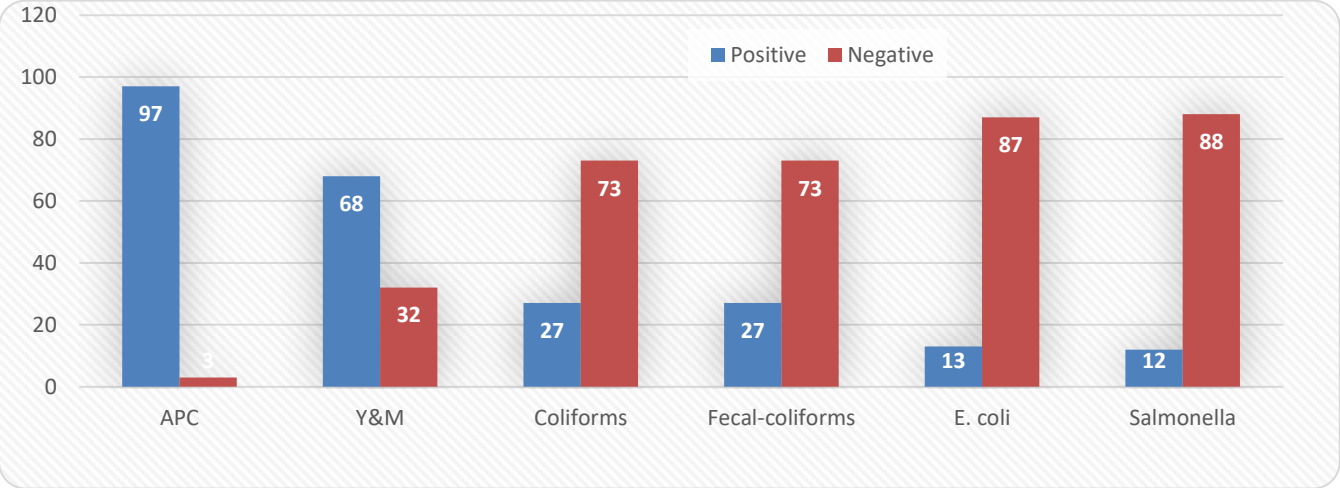

Figure 2: showing positive and negative number of Y&M and bacterial isolates from broiler feed, x-axis shows Quantity, while Y-Axis representing Micro-Organisms.

Figure 3: Growth of yeast and Moulds (*S. cerevisiae*, *Mucor*, *Aspergillus* and *Penicillium* Spp.) on solidified DRBC agar plate.

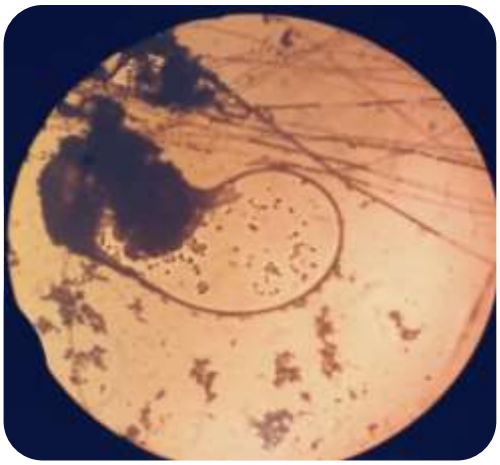

Figure 4: Microscopy of *Mucor* spp.

Statistical analysis and Frequency of Y&M

All the samples were analyzed in triplicates in order to determine any type of variation and then statistical analysis was performed. In case of Y&M, 68 samples out of 100 were detected positive and the mean deviation was 1267.47 while the standard deviation was observed as 2123.113. The analysis of isolates revealed a dominance of yeast, with 68 isolates comprising 100% frequency. *Mucor* spp. followed with 30 isolates, representing 44.11% frequency, while *Aspergillus* spp. accounted for 21 isolates, constituting 30% frequency. *Penicillium* spp. were also prevalent, with 50 isolates, making up 73.52% frequency. These findings provide insights into the composition and distribution of different genera among the analyzed isolates Table 1.

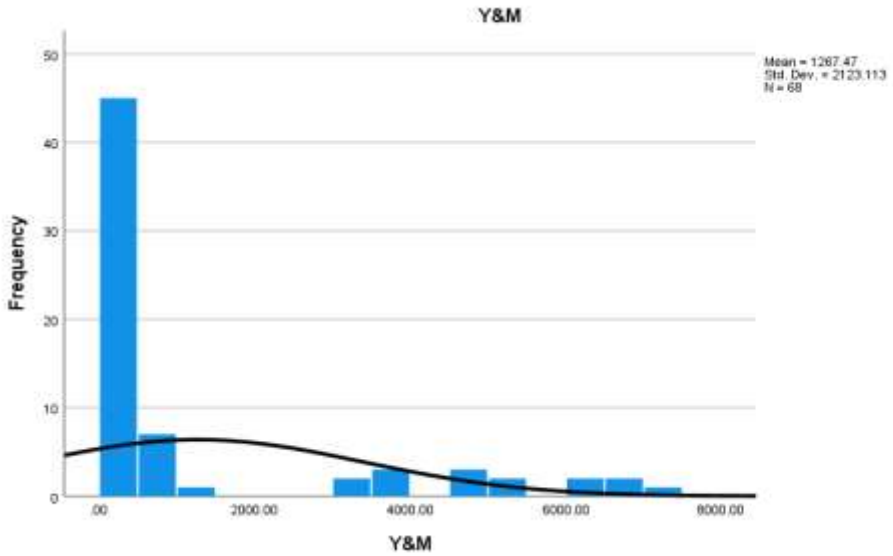

Figure 5: Showing the statistical analysis of Y&M.

Table 1. Frequency of isolated Y&M strains from poultry feed samples

Genus	No of Isolates	Frequency %
Yeast	68	100%
Mucor spp	30	44.11%
Aspergillus spp	21	30%
Penicillium spp	50	73.52%

Analysis of Bacteria

The Analysis of broiler feed samples revealed various microbial contaminants, including APC, coliforms, fecal coliforms, *E.coli*, and *Salmonella* spp. and suggests continuous monitoring to ensure food safety. Feed samples were cultured on general media for aerobic plate count, and colonies from these media were picked and inoculated into selective media for sub-culturing and identification of particular organisms (Onajobi et. al., 2023).

Aerobic plate count (APC)

The graph represents the number of samples showing APC. There are five categories on x-axis which indicates the number of colonies produced on PCA i.e. plate count agar (figure 7) and on y-axis, samples are mentioned. Out of 100 samples, 15 samples gave aerobic plate count between 0-5000 (1st category), 2nd category contained 49 samples, there were few samples that lied in 3rd and 4th categories, 9 samples were in range between 20000-25000 (5th category) and the remaining samples produced colonies more than 25000 (6th category).

Statistical Analysis of APC

In case of APC, around 97 samples gave positive results out of 100. Their mean deviation was 32656.32 and standard deviation was obtained as 51912.07.

E. coli,* Coliforms and fecal coliforms, *Salmonella spp

For the determination of bacteria, the samples were cultured on general and selective media. The said plates were incubated for 24h. On the next day, growth was observed and recorded. Further, gram staining and biochemical tests were performed to identify the bacteria. Results are listed in table 2 and 3.

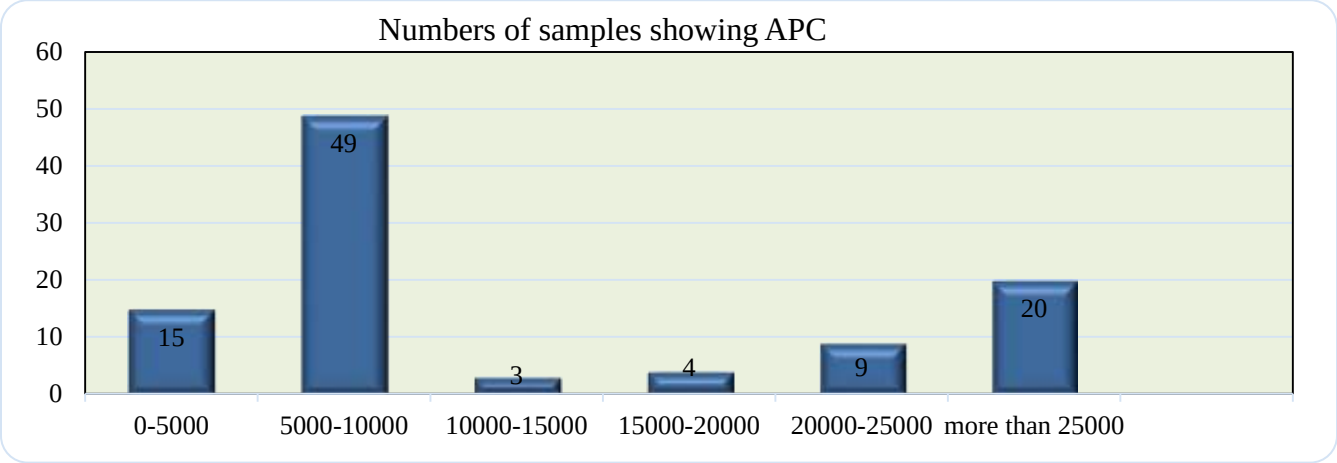

Figure 6: Aerobic plate count in feed samples in six categories.

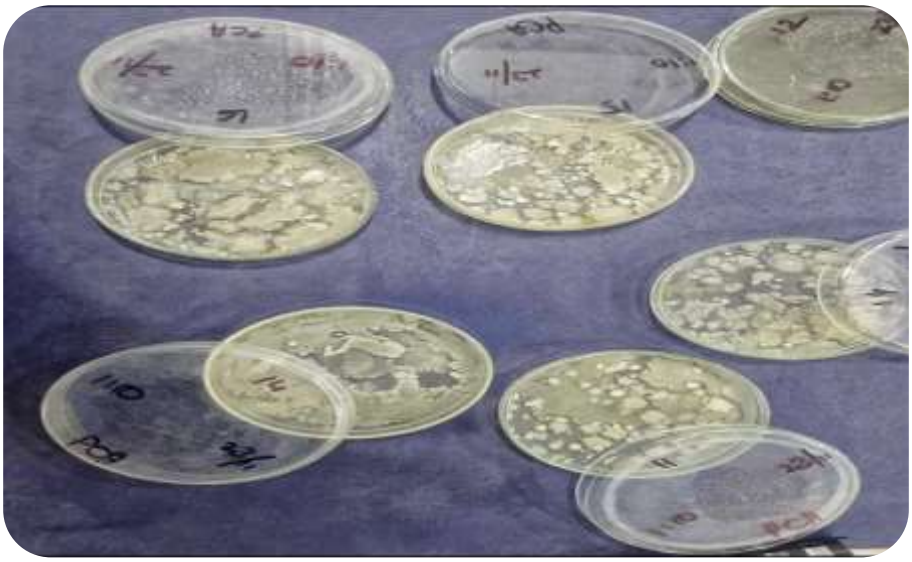

Figure 7: Growth on plate count agar (PCA).

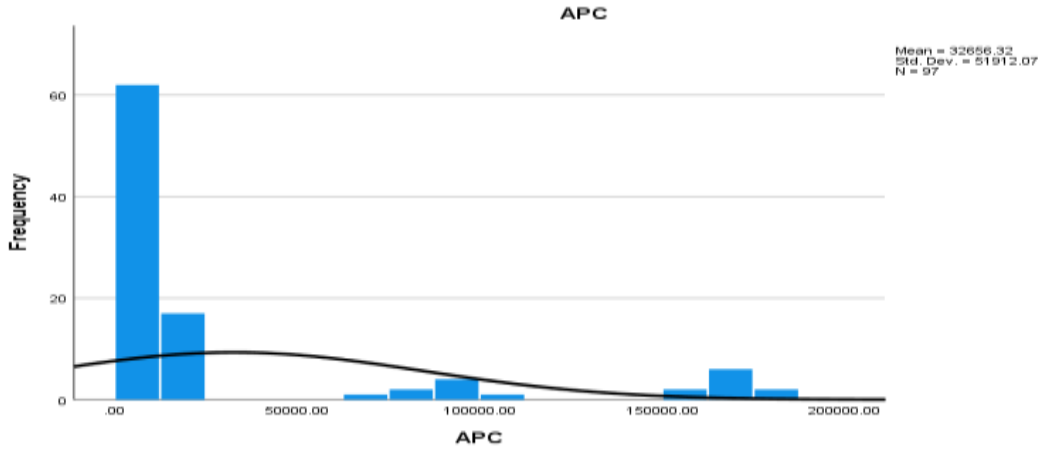

Figure 8: Statistical analysis of APC.

Table 2: Cultural and Morphological characteristics of detected bacteria in poultry feed samples.

Bacterial isolates	Morphological characteristics	Cultural characteristics
E.coli, Coliforms and fecal coliforms	smooth, circular, greenish black color colonies with metallic sheen were observed on EMB (figure 9)	Gram negative rods were observed (figure 10)
Salmonella spp	black colonies with slightly translucent zone were observed on XLD (figure 11)	Gram negative pink-red medium sized rods were observed

Table 3: Biochemical results of detected bacteria in poultry feed samples.

Tests	*Coliforms and fecal coliforms*	*E.coli*	*Salmonella*
Motility	+	+	+
Indole	+	+	-
MR	+	+	+
VP	-	-	-
Citrate	+	-	-
Gas	+	+	-
Lactose	+	+	-

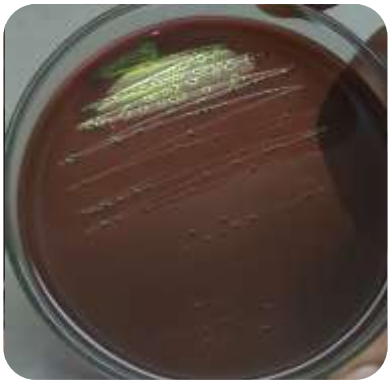

Figure 9: Green metallic sheen produced by *E. coli* on E.M.B.

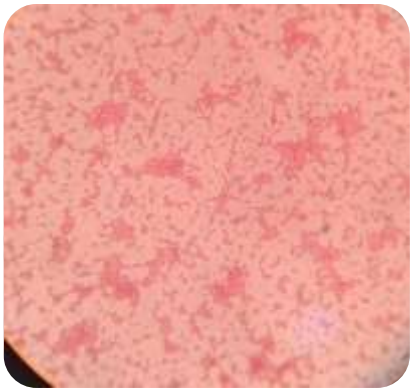

Figure 10: Gram negative rods of *E. coli*

Figure 11: Blackish colonies produced by *Salmonella* spp on XLD.

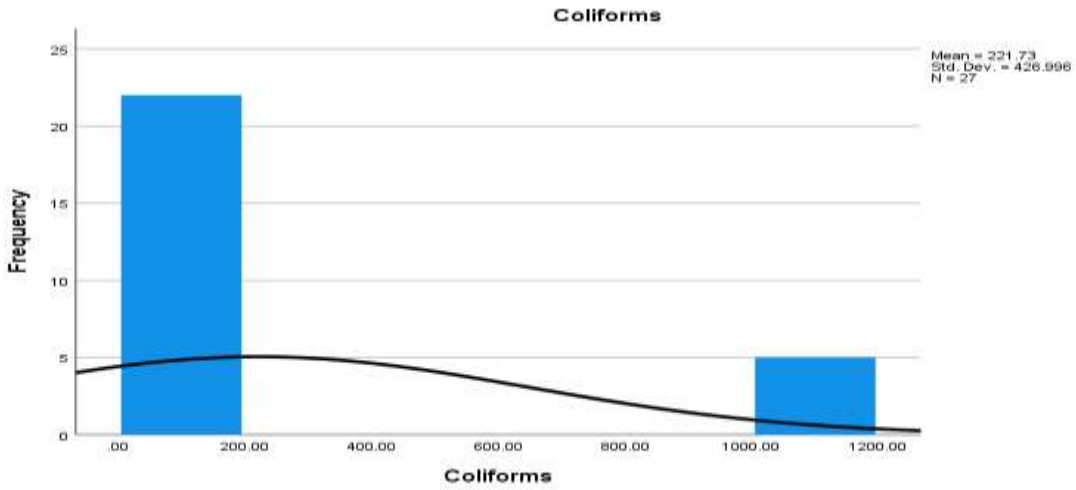

Figure12: Statistical analysis of Coliforms.

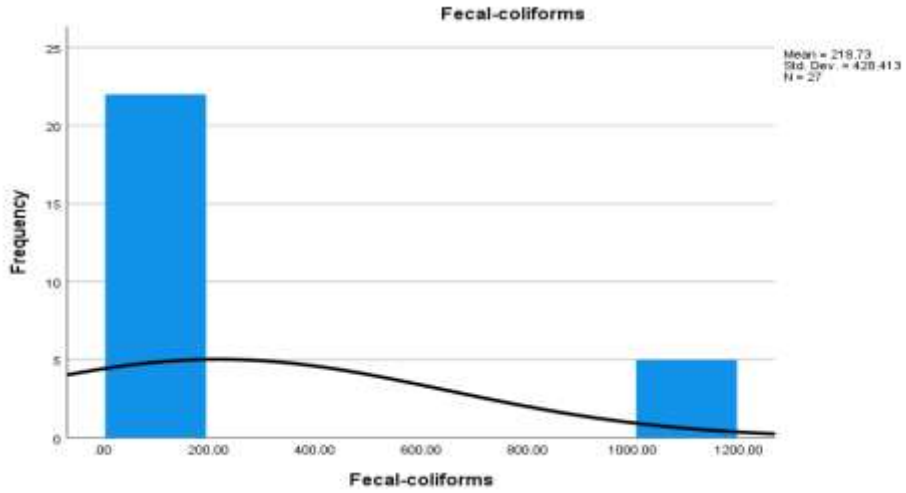

Figure 13: Statistical analysis of fecal-coliforms.

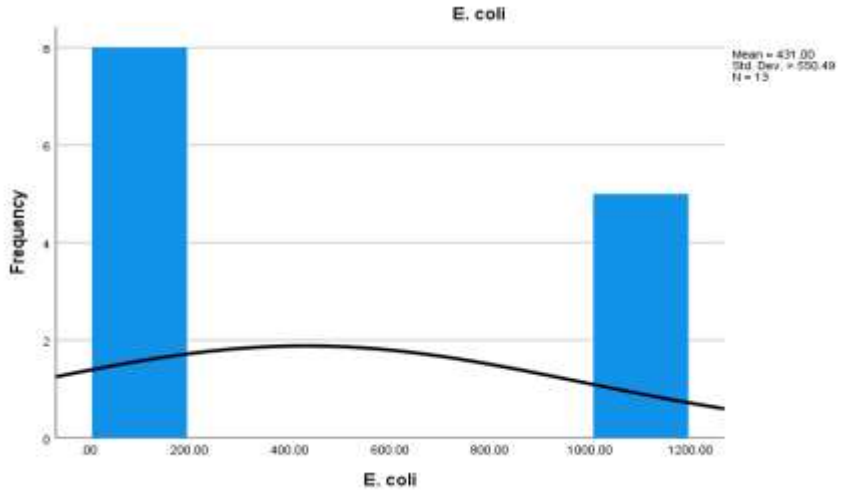

Figure 14: Statistical analysis of *E.coli.*

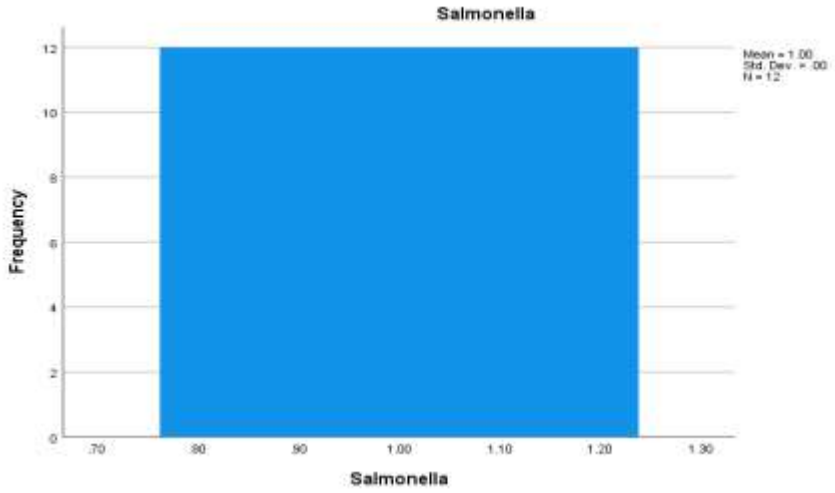

Figure 15: Statistical analysis of *Salmonella*.

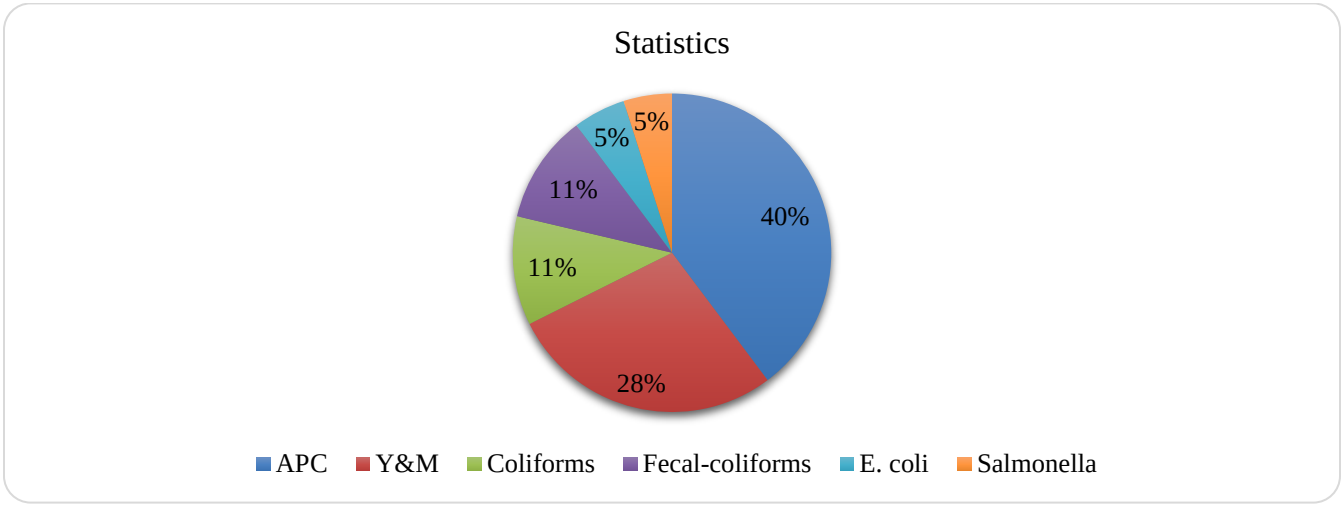

Figure 16: Graph representing detected microbial contaminants in feed samples in overall percentage (%) form, indicating high APC as compared to other contaminants.

DISCUSSION

Present study's objective was to investigate, microbial contamination existing in commercial feeds of broilers, in the Karachi region. The poultry industry has faced significant challenges, particularly health issues such as cholera, salmonellosis, Newcastle disease, *E. coli* infection, and coccidiosis, which have inflicted significant damage on broiler and broiler breeder flocks (Moryani, 2020). Microbial contamination prevailed throughout the year in the poultry feed. The occurrence of toxicogenic fungal / microbial contamination in raw materials and in finished broilers feed is sensitive to factors, such as feed-type, conditions of storage geographical location and season, with more or less humidity, temperature fluctuations, use of certain pesticides over crops/ ingredients used may impact grains moisture contents, quality, genetic factors and microbial populations. However, the highest bacterial contagion, diseases and total viable count due to high humidity and temperature documented from April to September Yunus et al and June to August by Shajeela et al., 2021 respectively.

One hundred samples of broiler feed, procured from diverse poultry farms and commercial feed markets in the vicinity of Karachi. A rigorous analysis was conducted, focusing on the identification of microbial entities, such as Salmonella spp, coliforms, yeast and mold (Y&M), aerobic plate count (APC), and fecal coliforms. Microscopic analysis confirmed

the presence of four distinct species: Yeast, *Mucor* spp., *Aspergillus* spp., and *Penicillium* spp.

To quantify yeast and mold populations, a dilution procedure was employed, and subsequent to dilution, the samples were plated onto the surface of solidified D.R.B.C. agar-plates, because of its distinctive features include the inhibition of rapid mold growth, leading to the formation of smaller, well-defined colonies. Further, its commercial availability and relatively low cost, enhance its utility in fungal enumeration (Tournas et al., 2007).

Among all the examined samples, Y&M growth was evident in 68 samples, while 32 samples exhibited no signs of growth. To substantiate this growth, microscopic analysis was conducted, confirming the presence of four distinct species, based on their cultural and morphological characteristics: Yeast, *Mucor spp.*, *Aspergillus spp.,* and *Penicillium spp.* (table 04). Identification of mycotoxigenic fungi traditionally relies on assessing colony morphology, encompassing physical aspects, and considering fungal-hyphae and spores microscopically (Alsalabi et al., 2023). Yeast and *Penicillium spp.* exhibited higher frequencies compared to other species, with yeast and *Penicillium spp.* at 100% and 73.52%, respectively. *Mucor spp.* and *Aspergillus spp.* showed frequencies of 44.11% and 30%, respectively.

Previous studies have consistently explained that the predominant flora in the majority of feeds belongs to the *Aspergillus* and *Penicillium* genera, and produce, OchratoxinA (OTA), a well-known nephrotoxic mycotoxin, may induce intestinal aging through calcium overload, oxidative stress, inflammation and toxicological effects (Wang, 2024). The prevention and reduction of (*Aspergillus*) are highly important in maintaining quality-control of poultry feed, as the production of aflatoxins can occur during the process of converting raw ingredients into finished feed (Seyed Soheil Ghaemmaghami *et al.* 2024). Various microbial contaminants were identified, including APC, coliforms, fecal coliforms, *E.coli*, and *Salmonella* spp. In results, APC was detected in 97 out of 100 total samples, with recorded counts exceeding 300 C.F.U. /g). Gross aerobic counts (TAC) serve as an indicator with implications for processing hygiene and storage quality. The observed elevated coliform levels may stem from potential sources of cross-contamination, including farm workers' hands during feed mixing, farm rodents and reptiles, live bird droppings, or environmental dust (Tahiru Mahami, 2019). *E. coli* was identified in 13 out of 100 samples, with distinct features of evenly rounded, greenish-black colonies having metallic-sheen. The occurrence of *Salmonella* was noted in 12 out of 100 samples, suggesting a relatively low *Salmonella* frequency in poultry samples.*Salmonella* prevalence in various animal feeds has declined from 18.2% in 2002 to 8.0% in 2009 (Kazimierska, 2021). According to Li et. al. (2012), there has been a decline in the overall *Salmonella* prevalence in various animal feeds, Salmonellosis, the second extremely reported illness publically, poses a significant risk in pet foods, as emphasized by (Kazimierska, 2021). Interestingly, *Salmonella spp* were not detected in the examined complete feed mixtures, as reported by Pavlovic et al. (2019). This suggests that *Salmonella* prevalence in poultry feed may vary, and continuous monitoring is crucial to ensure food safety.

All the samples were run in triplets, as it is a widely adopted practice in microbiology because, it enables statistical significance by allowing researchers to discern whether observed variations are statistically significant or merely a result of chance. Additionally, replicating experiments enhances the precision and accuracy of results, providing a more robust dataset for drawing reliable conclusions. This approach also serves as a form of internal quality control, helping identify any discrepancies in the results. Furthermore, replicates help mitigate experimental errors and account for the inherent variability in biological systems.

Poultry feeds incorporate essential nutrients for the overall well-being and optimal development, supporting meat and egg production in birds. However, if faulty components are used without following sanitary precautions, improper storage and feed is not examined in a lab, it might have an impact on poultry production and spread a variety of diseases that harm both humans and animals (Esan, 2024). The *Enterobacteriaceae* family contains the majority of the bacteria linked to contaminated feed. *Salmonella*, in particular, is responsible for a number of acute and chronic illnesses, including pullorum, avian arizonosis, typhoid, para-typhoid, plague, shigellosis, and many more. Avian pathogenic *Escherichia coli* (APEC) is responsible for various extra-intestinal illnesses in chickens, such as aero-sacculitis, poly-serositis, and septicemia. Additionally, several bird illnesses, considered potentially contagious to humans, and because of zoonotic importance pose significant public health risks and economic burdens in many countries.

CONCLUSION

The study explored the potential occurrence of microbial organisms, specifically Salmonella, E. coli, yeast, and

molds, in poultry feeds may be a source of salmonellosis, colibacillosis, mycotoxicosis hence causing financial losses because of treatment costs, stress and drop in production and significant burden on farmers pocket and in long run industrial and country's economy. These entities can pose substantial health risks to poultry and may carry the potential for transmission to humans, resulting in considerable low production rate or not desired growth or expected results of broilers by remaining under-weight after consuming good quantity of feed they may have low Feed consumption Ratio (FCR) and imposing a burden on public health because of its food-borne zoonotic importance, further use of antibiotics for treatment of infected birds may be a source of Anti-Microbial Resistance. Buying high quality ingredients, Aseptic handling, hygienic production process of commercial broiler feeds and proper silos and storage facilities because microbial contaminants in chicken feed can be transmitted at any stage during production, storage and supply, so biosafety and biosecurity measeures should be strictly adopted, further regular check and balance for ingredients through Research and development labs must be ensured for minimizing the contaminants.

LIMITATION AND FUTURE ASPECTS

Due to limited resources, finance and time, comparing broiler and layer feed was not possible. Commercial feed producers and poultry farm owners may not want researchers to interfere with their business. However, scientific research has made the industry what it is today, keen interest of all stakeholders with their potential in supporting and financing Continuous scientific research and collecting latest data, microbial load evaluations, may help in Sale and Purchase of high quality ingredients with proper moisture and bacterial contents and after examining in Research and Development Labs without compromising on quality, may help and guide accordingly for prevention from salmonellosis, collibacilosis, mycotxicosis and other losses, and may help in better Productions, increased hygiene and food-safety, supervised antimicrobial usage after sensitivity tests and proper dosage for the treatment of birds may results in decreasing drugs resistance, health and economic stability, industry's growth and hence prosperity of the beloved motherland, as it is considered as one of the largest industries of our country and playing its vital role in providing jobs, business opportunities and poverty eradication.

CONFLICT OF INTEREST

The authors declare that there is no conflict in the publication of this article.

AUTHOR'S CONTRIBUTION

All the authors contributed equally in the manuscript.

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