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Molecular diagnosis of hen eggs microbiota

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Abstract

The study was conducted in the Kingdom of Saudi Arabia from 2020 and 2022. The identification, characterization, and evaluation of microbes found in hen eggs was done and it was found very important to prevent contamination caused by various harmful pathogenic microbes. It was found that contaminated eggs harbor various harmful microbes which affect health due to multiple infectious diseases. Hen eggs contain a wide variety of microbes, and several distinct approaches were utilized as well as available for achieving detailed pathogenic information. The information obtained is highly essential for people who consume eggs as a food product. It is of the utmost importance to protect people from getting sick due to the consumption of contaminated eggs or eggs from chickens that have been infected by various harmful pathogens. During the experiment, we found that eggs were contaminated directly or the chicken that laid the egg was contaminated. Using molecular genetic analysis, it is possible to detect pathogenic and non-pathogenic contaminations in eggs. During present studies, the cutting-edge molecular techniques of 16S rRNA gene sequencing technology were used to carry out the objective of performing a molecular identification of the microbial communities infecting eggs. The present research is aimed at determining whether the microbial communities in hen eggs are harmful to humans. The results further indicated most bacteria have the potential to cause illness in humans including *Escherichia fergusonii, Salmonella enterica, Pseudocitrobacter faecalis, Yakenella regensburgei,* and *Erwinia pyrifoliae.* Further, research suggested that eggs need to be properly cooked and thoroughly washed to eliminate the possibility of consuming infected eggs.

Keywords: Eggs, Hen, DNA, 16s rRNA, PCR

1. Introduction

Currently, eggs are considered extremely expensive food items, even though they are an essential component of a balanced diet consisting of the most vital nutrients required for good health [1]. Eggs are consistently ranked among the top 10 least expensive food items derived from animals. To minimize the likelihood of acquiring foodborne infections and food poisoning, the consumption of fresh and hygienic eggs is important and calls for an increased level of care and attention to be exercised throughout the processes of egg-based food preparation and their handling [2]. Salmonella is the most significant pathogen found in eggs, capable of infecting humans and inducing severe illness. Consuming raw eggs, despite their apparent freshness and excellent condition, increases the risk of infection transmission, therefore eggs must be cleaned, cooked, and preserved before consumption. Eggs are a cheap source of proteins important for health, which is one of the numerous reasons for the widespread consumption of eggs across the world [3]; however, they are frequently linked to outbreaks of food poisoning because of the widespread bacterium spectrum that has the potential to be harmful, especially in European countries where there are over one hundred thousand cases of Salmonella infections every single year [4]. It is imperative to implement efficacious preventive measures to mitigate the likelihood of contracting infections caused by a multitude of organisms. Salmonellosis is classified as a form of food poisoning that is contracted through the ingestion of uncooked or undercooked eggs [5]. Molecular biology has facilitated the identification of a substantial quantity of genetic variations within DNA sequences, which are traced back to specific regions of the genome. The molecular markers-based approach is crucial in assessing the degree to which discernible phenotypic variations exist in DNA, as it facilitates the elevation of genetic variations [6]. Molecular markers are important to determine whether or not DNA has been altered in the genome mapping, however, the individual genotyping analysis of banding strips helps to determine traits of interest [7]. Identification of DNA polymorphism has become possible due to rapid development of the PCR [8] and diverse types of molecular DNA research have become possible. The sequencing of 16S rRNA and the application of RAPD techniques are both necessary components of molecular diagnosis and molecular determination of egg microbiota [9]. Since, molecular diagnosis of microbiota is essential for the development of genetic markers, which not only proved

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useful inputs in the process of selecting good and potent animals (hens) and their byproducts but also marked a significant step forward in the study of egg genetics [10]. The discovery of DNA markers in the past two decades has greatly facilitated the process of enhancing the genetic composition of farm animals [10]. The ongoing work employs a diverse range of molecular and microbiological techniques for identification, including the sequencing of the 16S rRNA gene, to accurately identify, categorise, and diagnose pathogenic bacteria found in chicken eggs [11]. Further, this investigation was also carried out to determine whether or not the eggs were contaminated with harmful bacteria.

2. Material and methods

2.1. The Isolation of Bacteria

For the isolation of microbiota, eggs were collected from several stores located all over Saudi Arabia. After incubating the egg samples for about 72 hours, at 30 degrees Celsius; 50 aliquots of 100 ml fluid were shifted onto nutrient agar (NA) plates, and plates were serially diluted using sterile distilled water. This was performed till the final concentration of the sample was prepared. After that, the plates were examined for more detail with additional molecular and microbial tests. The growth patterns of the colonies that were formed by a wide variety of unique microorganisms were examined in detail using an Olympus microscope. The bacterial isolates were suspended in glycerol at a concentration of 20%, and the resulting mixture was chilled to -80 degrees Celsius in the freezer. Further, the method used by [12], was followed for the determination of various microbial agents in poultry eggs.

2.2. Identification of bacteria using various biochemical techniques

2.2.1. Gram-staining

Gram-staining techniques were used for bacterial identification followed by the method given by [13].

2.2.2. Oxidase Test

To carry out the Oxidase test, tetramethyl-p-phenylenediamine dihydrochloride was mixed with water to create a solution that had a concentration of 1% (see method given by [14]. After preparing tetramethyl-phenylenediamine solution, samples were poured onto a strip of filter paper (Whatman No-1) and later placed in a petri dish. The petri dish was later incubated at room temperature for 24 hours under RH 75%. The Petri- dish was later put into an incubator set at 37 degrees Celsius for 24 hours. The area of the strip that had been impregnated was swabbed with a bacteria loop that had been grown on NA medium nearly 24 hours before its use. Sixty seconds after the beginning of the test, a purple color appears on the screen indicating that the test was completed and successful.

2.2.3. Catalase assay

After the growth of microbes in the NA medium for 24 hours, the catalase activity of the bacteria was measured using the method given by Iwase et al. [15]. This was done before the analysis began, and immediately followed by a mixing loop of each bacterial culture with a drop of hydrogen peroxide, and the resulting mixture was placed on a clean glass slide. The slide prepared was observed under a microscope and gas bubble formation was observed more clearly. In the presence of catalase, a positive test result was achieved once gas bubbles were found, indicating that multiple enzymes were present in the samples**.**

2.3. Isolation of genomic DNA from Bacteria

DNA was successfully purified by utilizing a QIAamp genomic DNA purification kit [16], and strict instructions were followed that were provided by the manufacturer. The purification process was done accurately and after culturing, each bacterial strain was kept for 24 hours in nutrient broth (NB). Each strain of solution 1.75 milliliters long was poured into a tube and centrifuged at a rate of 13,000 RPM for five minutes and all results were recorded. After removing the supernatant from the test tube, 180 ml of enzyme lysis buffer was added, and later it was shaken for 10 to 20 seconds. After adding 25 ml of proteinase-K and 200 ml of the alkaline buffer to the mixture, it was placed back into the incubator and maintained at 56 degrees Celsius for an additional 30 minutes. Following the addition of 200 ml of ethanol with a concentration of 100%, the solution was centrifuged at 13000 RPM before being transferred to the mini-column for further processing. After the sample had been centrifuged for one minute at 13,000 RPM, the supereminent was later removed and the column was washed with 500 liters of AW1 wash buffer, and later dried.

The results were recorded after adding 500 ml of the AW2 washing buffer, and the mixture was spun for three minutes at 13,000 RPM. After that, 120 ml of water was added without excluding the addition of nucleases, and centrifuged at 13,000 RPM. After it had been isolated, the DNA obtained was allowed to cool down to a temperature of -20 degrees Celsius before it was analyzed.

2.4. Polymerase Chain Reaction-Amplification 16s rRNA

To perform amplification of the 16S rRNA gene, primers with the sequences 5'- CAGCGGTACCAGTTTGCTGCTCAG-3' and 5'- CTCTCTGCAGGCTACCTTGTACGACTTT-3' were utilized. These primers were complementary to one another and after denaturation for four minutes at 94 degrees Celsius, the amplification cycle consisted of thirty iterations each of which lasted one minute at 94 degrees Celsius, thirty seconds at 58 degrees Celsius, one minute at 72 degrees Celsius, with ten minutes of extension and one minute at 4 degrees Celsius. The denaturation cycle was preceded by an amplification cycle that lasted for four minutes and the amplified genes obtained were found under UV light and electrophoretically separated on an agarose gel. The 16S rRNA gene segment that had been amplified was later subjected to sequencing.

2.5. Phylogenetic analysis

Basic Local Alignment Search Tool (BLAST) was used to analyze 16S rDNA sequences of the bacteria. The results of this analysis were later compared to sequences and stored in NCBI GenBank (BLAST). A phylogenetic tree was constructed with the help of the neighbor-joining method utilizing the MEGA (7.0.26) software program. As a direct consequence of this, the genesis of the tree was finally made attainable. The Kimura 2-parameter model allowed us to perform the calculations necessary to determine the distances in evolution.

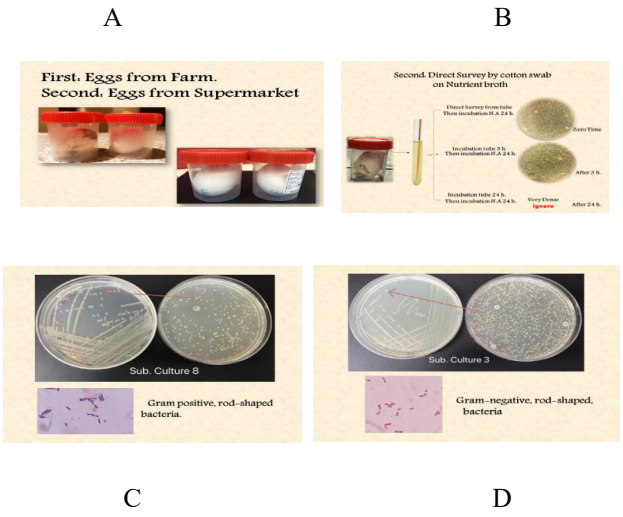

Fig. 1. The Eggs samples A, B, culture media and gram staining: C, gram-positive, and B, Gram-negative.

2.6. Statistical analysis

The BLAST tool was employed to examine the 16S rDNA sequences of the bacteria. The results of this analysis were later compared with sequences and stored in NCBI GenBank (BLAST). A phylogenetic tree was constructed using the neighbor-joining method included in the MEGA (7.0.26) software program and ANOVA analysis.

3. Results

A total of 60 samples were taken from 10 different retail establishments to investigate the possibility of pathogenic bacterial strains being present in eggs. Various egg samples were collected to do so as mentioned in the Material and Method section. The media such as, blood, chocolate, and the MacConkey medium were used for pure bacterial colony growth to bring desired outcomes. Microbes were classified into appropriate categories and various characteristics of bacterial isolates and the results of their biochemical examinations were taken into consideration. The four bacterial isolates that were chosen (A, B, C, and D) were displayed with unique morphological characteristics, such as gram-positive and gram-negative staining. These characteristics were shared by different types of bacteria and all characteristics were found to vary in each isolate as shown in Fig. 1.

Cloning the 16S rRNA gene and sequencing

Cloning the 16S rRNA gene and sequencing are two crucial steps in the process of determining the molecular and genetic structure of bacteria that were present in eggs. For bacterial characterization and detection, bacterial isolates were analyzed at the molecular level with the assistance of the QIAamp genomic DNA purification kit. The high-quality genomic DNA was successfully extracted during the procedure. According to the findings of the present study, the amount of genomic DNA extracted from a specific volume ranged anywhere from 60 ng/l to 140 ng/l. Further, By employing universal forward and reverse 16S rRNA primers in a Polymerase Chain Reaction, ribosomal DNA was successfully amplified and DNA fragments were generated that were approximately 500 base pairs in length. The PCR products were amplified and separated on an agarose gel-1% and finally analyzed using a UV transilluminator. The extent of the potential diversity that is present within the amplified regions was determined and later accomplished by making use of phylogenetic analysis as a methodological component of the present experiment. Egg samples were analyzed, as shown in Figures (2-7) and Tables (1 and 2), have the potential to contain a wide variety of pathogenic bacteria especially *Escherichia fergusonii* and *Salmonella enterica sub spp. Pseudocitrobacter faecalis, Yakenellaregens burgei, Erwiniapyrifoliae*, and *Bacillus subtilis*.

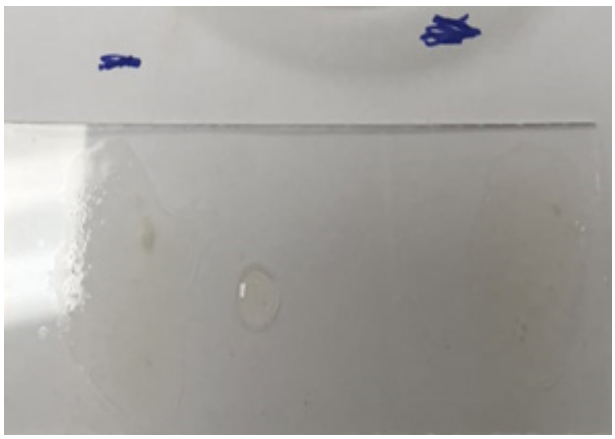

Fig. 2. H200729-020-C23-M-B12-11492R (*Lactobacillus pentosus*-gene) gram positive/ catalase negative/ oxidase negative.

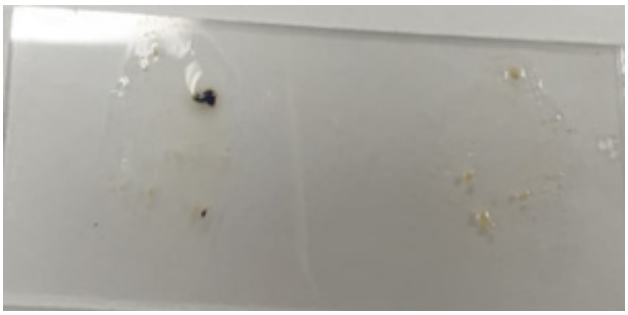

Fig. 3. *Bacillus subtilis* strain A2 16S ribosomal RNA gene, partial sequence, gram-positive/catalase positive/oxidase variable, H200729-020-A15-M-A08-1492R.

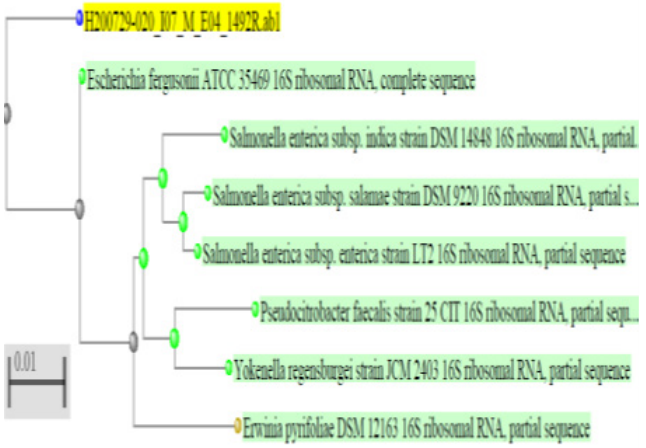

Fig. 4. Phylogenetic analysis of bacteria isolates, such as *Escherichia fergusonii; Salmonella enterica* subsp; *Pseudocitrobacter faecalis; Yakenellaregens burgei,* and *Erwinia pyrifoliae* from different eggs samples based on partial nucleotide sequences of 16S rRNA gene. The tree was constructed using the Neighbor-Joining Method (NJM) (Saitou and Nei. 1987).

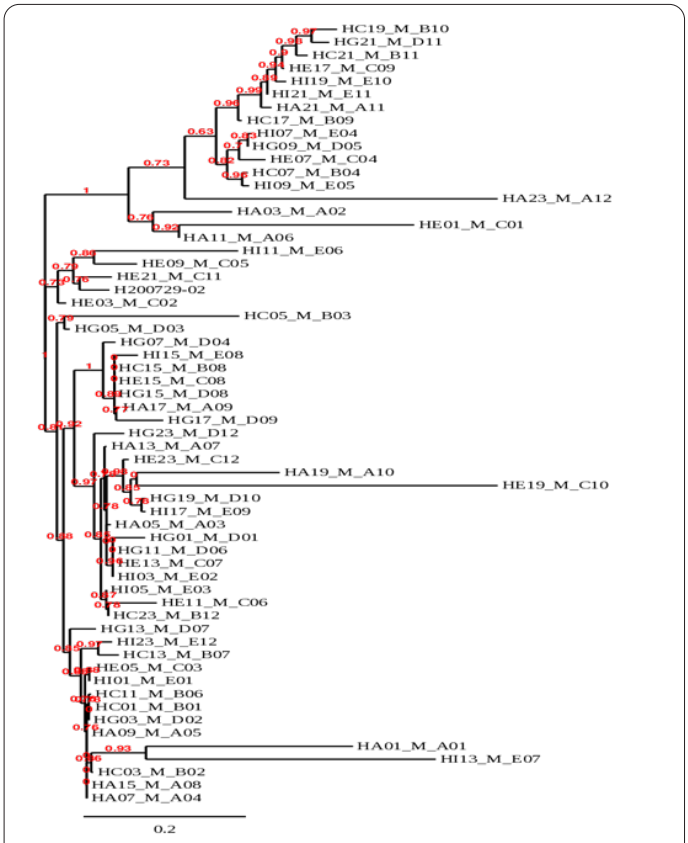

Fig. 5. Based on Partial Nucleotide Sequences of the 16S rRNA gene, phylogenetic analysis was performed on all bacterial isolates from egg samples using the LIAMM method to build the tree (Challa and Neelapu, 2019).

4. Discussion

Identification, characterization, and evaluation of microbes in eggs are essential to prevent harmful germ contamination and their spread to human beings. There are many ways to obtain pathogen information from hen eggs that are important for egg consumers. A huge literature is available demonstrating that bacteria can contaminate both the eggshell and the egg contents [15] even when eggs are kept in a clean environment. Eggs laid by hens have an increased risk of harboring pathogenic bacteria due to myriad ways of contamination. Bacteria are horizontally transmitted into chicken eggs while the eggs are still inside the oviduct of the hen before being laid [17]. However, infections were found spreading vertically from one bird to the next in a chain of birds while they were still laying eggs, especially when birds are close to one another. *Salmonella* is one of the many types of bacteria that is present in the food canals of hens, in association with other bacteria such as *E. coli.* Eggs are laid *via* the cloaca, which is the final part of the reproductive system, urinary, and digestive systems, and there are higher chances of infection from bacteria. This occurs because the cloaca is the terminal point of the reproductive, urinary, and digestive systems of the body, and sources of infection are multiple [18]. It has been found that eggs, from both exterior and interior sources, contain trace amounts of the byproducts that are produced by harmful bacteria

In the present investigation, nearly 73.25 percent of *Staphylococcus species* were found in chicken eggs in comparison to *Staphylococcus aureus, E. coli,* and *Klesbacter* spp. contributing 3.25 8.75, and 10.5 percent, respectively. There have been many studies that have resulted in many different findings concerning the incidence of these pathogens, which may be partially attributable to differences in sample sizes as well as the procedures that were utilized to randomly select eggs for bacterial examination.

According to some researchers, there is not even a remote possibility of *Salmonella* contamination in eggs obtained from grocery stores or roadside stands, however, Lee et al. [19] included that microbiota in eggs is the same as we found in current studies. Ansah et al. [20] also found that egg samples from Northern Ghana contained *Staphylococcus* and *E. coli*, which confirmed our findings about the prevalence of these bacteria in egg samples.

Present results were also supported by an independent investigation by Sharan et al. [21] in which it was discovered that egg samples from Nigeria contained *E. coli, Staphylococcus aureus,* and a *Klebsiella* species. All three bacteria are known to cause food-borne illness, while *Coliform* bacterium infections are frequently brought on by contamination with feces as well as a general lack of cleanliness in the area [21]. Eggs that are either sold in retail stores or come from poultry farms that do not adhere to adequate hygiene standards frequently have bacteria such as *E. coli, Klebsiella* spp., and *Citrobacter* either on the surface or inside of them. Eggs that are not pasteurized also frequently have these types of bacteria and current findings are in line with the findings that were previously

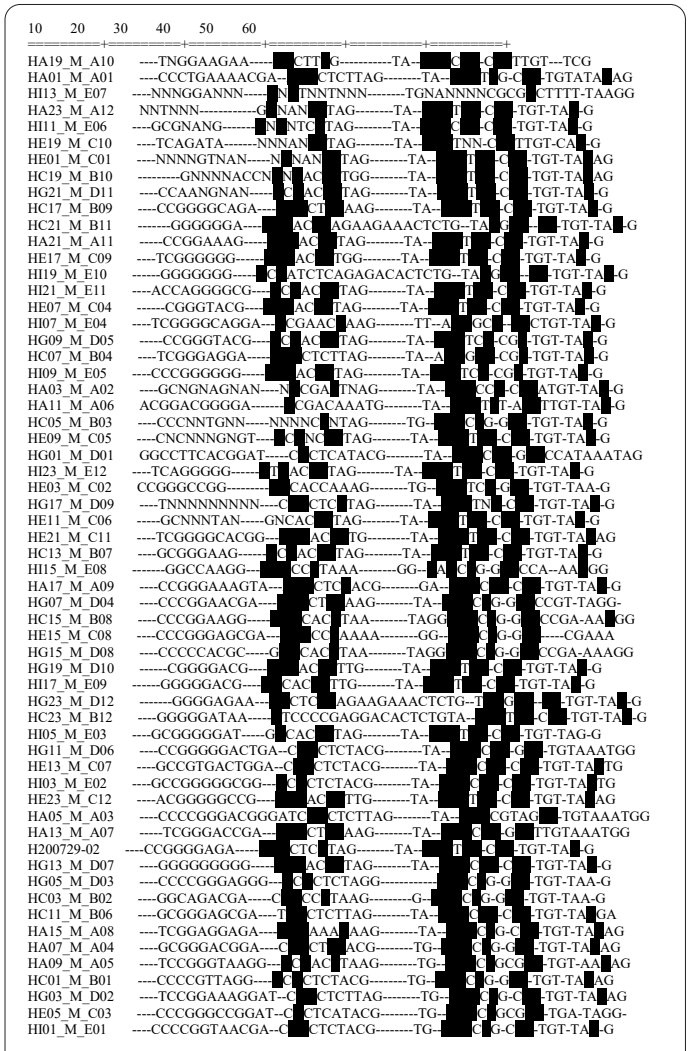

Fig. 6. G-blocks Results in 0.91b, input, FASTA processed file; Phylip, Clustal, EMBL format. Alignment is presumptively DNA, with total new positions equal to 209 (selected positions are underlined in blue).

Table 1. Relative abundance (%)of different microbes in eggs laid at different periods of the day.

Species	Morning	After-noon	Evening	Night
Escherichia fergusonii	7	50	10	8
Salmonella enterica	12	43	24	14
Pseudocitrobacter faecalis	21	37	22	20
Yakenella regensburgei	10	35	30	25
Erwinia pyrifoliae	18	38	23	21
Bacillus subtilis	15	40	21	24
*Lactobacillus pentosus*gene	18	45	23	14
Staphylococcus species	10	78	47	35
Staphylococcus aureus	24	50	2	2
Escherichia coli	9	12	8	6
Klesbacter spp.	12	14	9	7

Table 2. ANOVA of relative abundance percentage of different microbes in eggs laid at different periods of the day.

Timing	N	Mean	Std. Dev. (SD)	Std. Error (SE)	Degree of freedom	SS	MSS	F-state	p-value
Data summary					**ANOVA Summary**				
Morning	11	14.18	5.43	1.63	3 (between)	4738.60	1579.53	10.48	
Afternoon	11	40.18	17.83	5.37	40 (Within)	6024.17	150.60		0.00003
Evening	11	19.90	12.47	3.76	-				
Night	11	16.00	9.98	3.09	Total=43	10762.76	-		

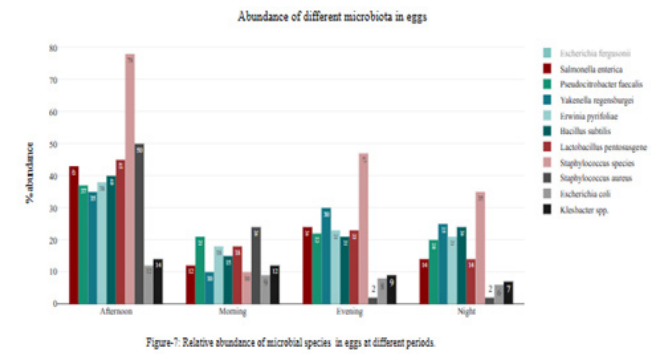

Fig. 7. Bar chart showing the relative abundance of various microbes present in eggs at different periods of the day.

obtained.

In this study, we also investigated whether there was a correlation between the time of egg collection and the level of bacterial contamination. Specifically, we investigated whether or not there was a correlation between the two. A significant factor that contributed to the outbreak was the presence of bacterial contamination at the collection site throughout the day when the eggs were being gathered' which led to the disease outbreak. The microbial load of the eggshell samples that were collected in the afternoon from all five farms and three marketplaces was slightly higher than the microbial load of the samples that were collected in the morning, and a high level of statistical significance was found (p>0.00003) (Tables 1 and 2, Fig. 7).

Since mesophile bacteria multiply more quickly when the temperature is between 25 and 40 degrees Celsius, afternoon samples have a higher microbial burden than the mornings and evenings due to temperature variations that range between 25 and 40 degrees Celsius. Even though the temperature varies from 10 to 25 degrees Celsius is a major factor in infection spread, confirmed the findings of [22,23] who worked on various pathogens of eggs and showed a good relationship and variability with changing temperatures.

In the current research, we employed different primers and reverse primer 1492R (5′-TASGGHTACCTTGTTAS-GACTT-3′) to target the entire 16S rRNA gene of egg bacteria. Figures 5,6, in 6 demonstrated the single-molecule real-time (SMRT) Bell sequencing libraries formed by PCR amplification, purification, quantification, and normalization. The genetic sequences of samples identified by barcode sequences are displayed in the findings (Figure 7). After the 16S rRNA Sequence, Data Preprocessing, Denoising, and Clustering were done to acquire the number of SeqNum sequences. Egg Sample community composition and abundance were assessed, and Lee et al. [24]conducted a detailed study on the microbiota profile of eggs and found *Riemerella anatipestifer, Klebsiella, and Escherichia/shigella* in egg samples. Similar studies were also done by Jin ey al. [25] that eggs have diverse microbial compositions which have a direct impact on their hatchability. Eggs are nutritious, but contamination by coliform bacteria and other dangerous organisms also causes major health issues. It has been found that coliform bacteria and the diseases it causes if not removed by boiling will cause huge health problems in human beings. Eggs contain a wide range of germs, which indicates that chicken farms and marketplaces have very weak hygiene standards. Dai et al. [26] found that eggs at grocery shops, restaurants, and refrigerators can spread *Salmonella* and other severe infections therefore it is strongly recommended that stringent health regulations be adhered to in chicken farms and markets. The findings of the current research may be incorporated into formulating public policy decisions regarding ways to lessen the incidence of bacterial contamination in farms that are used for the production and

distribution of poultry birds and eggs. Taking these points into consideration, strong measures will help to reduce the risk of foodborne illness. Further, present results will also help in deciding the consumption of neat and clean edible products obtained from commercial farms and markets.

5. Conclusion

After testing, eggs were found to *contain Escherichia fergusonii, Salmonella enterica subsp, Pseudocitrobacter faecalis, Yakenella regensburgei, and Erwinia pyrifoliae*. Eggs came from poultry farms and stores need to be kept clean and all hygienic measures need to be followed. *Bacillus subtilis* was the major pathogen in the egg specimen analysed for molecular investigations. Studies demonstrated that enhanced hygienic measures, notably clean equipment and adequate techniques, are necessary to prevent egg contamination. Eggs may thoroughly be cleaned to remove all germs. To avoid egg pathogens, these steps should always be taken. Molecular genetic analysis enables the differentiation between pathogenic and non-pathogenic egg contamination; and the state-of-the-art of molecular methodologies involving 16S rRNA gene sequencing technology were employed to accomplish the aim of identifying the microbial communities that infect eggs at the molecular level.

Interests that are at odds

No potential conflicts of interest were disclosed by the authors.

Declaration of contribution

Authors share in all steps in the work.

Data availability

All data that support the findings from this study are available from the corresponding author upon reasonable request.

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