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Microbiological Analysis of Commercially Available Poultry Feed in Pakistan

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TI performed experiments, formal data analysis and wrote original draft. SZ conceptualized and designed the experiments, supervised the experiments, contributed in data analysis and reviewed and edited the manuscript. FM reviewed and edited the manuscript. All authors read and approved the manuscript.

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ABSTRACT

Poultry feed industries are rising globally to meet the increasing demand of the growing population for protein. These feeds supply nutritious food supplements to ensure good growth and more eggs from the poultry. However, these feeds also support the growth of microbes that can cause different diseases in the poultry as well as in the consumers utilizing unprocessed and undercooked poultry products. Commercially available poultry feed samples in Pakistan were examined for contamination with different microbes (bacteria and fungi). Twenty-four feed samples were inoculated on different media to determine viable count, coliform count, *Staphylococcus* count, *Salmonella* and *Shigella* count. Different biochemical tests (Gram staining, catalase test, oxidase test and carbohydrate fermentation tests) and molecular method (16S rRNA gene sequencing) were used for the identification of microbes. The feed samples were found to be contaminated with *Salmonella enterica*, *Staphylococcus aureus, Enterobacter cloacae*, *Bacillus haynesii, Bacillus subtilis, Shigella* sp., *Escherichia* sp. and *Ralstonia* sp. Highest total viable count (7.07 ± 0.17 log CFU/g) was found in chick feed. S*almonella* count in the layer feed was recorded as 5.53 ± 0.36 log CFU/g and *Shigella* count in the broiler feed was 6.54 ± 0.78 log CFU/g. Coliform count was found in the range of 4.30 – 6.19 log CFU/g. *Staphylococcus* count was recorded in the range of 5.56 - 6.54 log CFU/g. Two feed samples were found to be contaminated with fungi, *Aspergillus niger* and *Aspergillus flavus*.

INTRODUCTION

The term poultry can be used for wide range of birds but in Pakistan, poultry generally refers to chickens (Krnjaja *et al*., 2008). Rapid population expansion in the 20th century resulted in increased demand of food that leads to the industrialization of different food sectors. About 40-45% of meat requirements in Pakistan are fulfilled from chicken. In Pakistan, poultry industry business first started in the 1960s and in 2018, it became the most organized branched of Agro-business, producing 18000 million eggs and 2250-million-kilogram chicken meat annually (Hussain *et al*., 2015).

With the development of the poultry industry, the production of poultry feed increased significantly and the industries offered different types of poultry feed either in the form of pellets or mash. Poultry feeds are formulated to meet the dietary requirements of chicken and can be categorized as chick feed, grower feed, broiler feed and layer feed. However, the safe food chain can be interrupted by the presence of microorganisms. Contaminated feed is a major source of transfer of these pathogens to poultry. These pathogens may become a source of diseases that include fowl typhoid, Newcastle disease, fowl pox diseases and other respiratory, or gastrointestinal diseases resulting in mortality of chickens. It is reported that human can become ill after consuming the chickens that were fed on contaminated feed (Yunus *et al*., 2009).

Different factors contribute to the contamination of feed. Different type of feed ingredients and animal products used in the poultry feed are a source of contamination (Lemme *et al*., 2004). Drying activity and storage of grains in poorly ventilated rooms also contribute to the contamination of poultry feed (Habib *et al*., 2015). Different microbes associated with poultry feed contamination include *Pseudomonas, Yersinia* (Waldroup, 1996), *Listeria, Streptococcus, Clostridium, Staphylococcus, Enterobacter, Proteus, Bacillus* (Bryan and Doyle, 1995; Obi and Ozugbo, 2007; Uwaezuoke and Ogbulie, 2010), *Salmonella* (Alshawabkeh, 2010; Uwaezuoke and Ogbulie, 2010), *Shigella* and *Escherichia* (Obi and Ozugbo, 2007; Chowdhury *et al*., 2011). Different fungi have also been

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associated with poultry feed i.e., *Aspergillus* (Cegielska-Radziejewska *et al*., 2013; Aliyu *et al*., 2016), *Rhizopus, Mucor* (Cegielska-Radziejewska *et al*., 2013; Okoli *et al*., 2007), *Penicillium, Fusarium, Stachybotrys* and *Claviceps* (Sobczak *et al*., 2016; Anifowose and Bakre, 2021).

Rapid identification of pathogens involve identification at the molecular level (Mandal *et al*., 2011). 16S ribosomal RNA gene amplification is used to identify the isolates, as it is present in all bacteria as a multigene family, its size is suitable for different kind of studies (Janda and Abbott, 2007) and it marks evolutionary distance and relatedness of organisms (Lagatolla *et al*., 1996). The present study was designed to check the poultry feed contamination in Pakistan with respect to viable, coliform, *Staphylococcus, Salmonella* and *Shigella* count and characterization and identification of the isolates by morphological (colony and cell characteristics), biochemical (Gram staining, Catalase, oxidase, carbohydrate fermentation test) and molecular means (16S rRNA gene sequencing). The feed samples were also checked for fungal contamination.

MATERIALS AND METHODS

Sampling

Total twenty-four samples (Chick feed, Grower feed, Layer feed, Broiler feed) were collected from Tollinton market (Lahore) and Pakpattan market (Pakpattan), factory areas in Lahore and rearing places of poultry in Pakpattan, Pakistan in sterilized bags and were processed in the laboratory.

Culturing

Different media; Nutrient agar (NA), Mannitol salt agar (MSA), *Salmonella-Shigella* agar (SSA), MacConkey Agar (MCA), Eosin Methylene Blue agar (EMBA) and Potato Dextrose Agar (PDA) were used for isolation of microbes. Samples were processed in duplicates. Samples (0.1 mL) were cultured from 10^{-4} dilution. Colonies were counted from 24 h incubated plates at 37°C and their colony forming unit (CFU/g) were calculated by using the formula:

CFU/g= Colony number × dilution factor/ volume plated (mL)

Morphological characterization

Morphological characteristics (color, shape, form, elevation, and margin) of isolated bacterial colonies were noted from 24 h incubated plates at 37 °C. Morphological characteristics of the fungal colony (color, surface, and texture) were noted from 3-5 days incubated plates of potato dextrose agar at 30 °C.

Table I. Total viable and coliform counts.

Biochemical characterization

Bacterial isolates were subjected to biochemical characterization using Gram staining, catalase and oxidase test. Sugar fermentation tests were performed with respect to glucose, maltose, sucrose and xylose (Holt *et al*.,1994).

Molecular characterization

DNA was isolated by using the method of Kronstad *et al*. (1983) and 16S rRNA gene was amplified by using the primers 27F; 5'-AGAGTTTGATCCTGGCTCAG 3' and 1492R; 5' GGTTACCTTGTTACGACTT 3'. PCR reaction mixture contained RNase treated genomic DNA (1.5 µL), 2.5 mM dNTPs (2.5 µL), 10 µM forward and reverse primer (1.25 µL each), 25 mM magnesium chloride (2.5 µL), IX PCR buffer (2.5 µL) and 2.5 units of Taq DNA polymerase (1 µL) in a 25 µL reaction mixture. The reaction mixture was incubated at 94°C for 5 min and then subjected to 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 90 sec; followed by one final cycle of 72°C for 10 min. PCR products were analyzed by using 1% agarose gel electrophoresis. The amplified gene was sequenced commercially and Blast tool of NCBI was used to find the highest similarity of 16S rRNA gene with related strains. The phylogenetic analysis was carried out using Mega X with bootstrap values (%) based on 1000 replications (Jukes and Cantor, 1969).

Statistical analysis

Total viable count (TVC), total coliform count (TCC), Total *Staphylococcus* count and total *Salmonella* and *Shigella* were statistically analysed using ANOVA and Duncan's multiple range tests using Costat (Snedecor and Cochran, 1980).

RESULTS

Among the twenty-four samples tested, highest TVC (7.07 ± 0.17 log CFU/g) was obtained for chick feed while the layer feed had the least contamination (6.22±0.06 log CFU/g). Grower feed had TVC value (6.93 ± 0.06 log CFU/g) (Table I). Highest TCC (6.19 ± 0.18 log CFU/g) was found for broiler feed on EMBA while lowest count (4.30 ± 0.45 log CFU/g) was recorded for grower feed (Table I). *Staphylococcus* contamination was higher in broiler and chick feed with mean log CFU/g values of 6.89 ± 0.65 and 6.56 ± 0.64, respectively while in grower feed, mean log CFU/g value was 5.56 ± 0.04 (Table II). Three samples of layer feed were found positive for *Salmonella* (5.53 ± 0.36 mean log CFU/g). *Shigella* was found only in broiler feed (33% samples) with average mean log CFU/g value of 6.54 ± 0.78 (Table II).

Sample	Total samples	Total viable count		Total coliform count			
				EMB agar		MacConkey agar	
		Positive samples (%)	log Mean CFU/g ± SD	Positive samples (%)	Mean log CFU/g ± SD	Positive samples (%)	Mean log CFU/g ± SD
Chick feed	6	100	7.07 ± 0.17^{b}	100	5.79 ± 0.73^{b}	66	5.40± 0.37^{b}
Grower feed	6	100	6.93 ± 0.06^{b}	100	4.30 ± 0.45^{a}	100	6.06 ±0.14^{c}
Layer feed	6	100	6.22 ± 0.06^{a}	100	5.99 ± 0.45^{b}	100	5.25±0.56^{b}
Broiler feed	6	100	6.34 ± 0.86^{a}	100	6.19 ± 0.18^{c}	83	4.53±0.74^{a}

± indicates standard deviation among samples. Values carrying the same alphabet are not significantly different at ($P < 0.05$).

Table II. Total *Staphylococcus*, *Salmonella* and *Shigella* counts.

Sample	Total samples	*Staphylococcus* count		Total *Salmonella* count		Total *Shigella* count	
		Positive samples (%)	Mean log CFU/g ± SD	Positive samples (%)	Mean log CFU/g ± SD	Positive samples (%)	Mean log CFU/g ± SD
Chick feed	6	100	6.56 ± 0.64^{b}	0	-	0	-
Grower feed	6	100	5.56 ± 0.04^{a}	0	-	0	-
Layer feed	6	100	6.25 ± 0.82^{b}	50	5.53 ± 0.36	0	-
Broiler feed	6	100	6.89 ± 0.65^{b}	0	-	33	6.54 ± 0.78

± indicates standard deviation among samples. Values carrying the same alphabet are not significantly different at ($P < 0.05$).

Morphological characterization results (color, shape, form, elevation and margin) of thirty-two different bacterial colonies isolated from twenty-four feed samples on different media (NA, MCA, MSA, EMBA, SSA) showed that colonies obtained on mannitol salt agar were yellow, circular, gelatinous, convex and entire; on eosin methylene blue agar, black circular, gelatinous, convex and entire colonies with green sheen were observed. On MacConkey agar, two types of colonies were found, one type being pink, circular, gelatinous, convex, entire and the second were light pink, punctiform, gelatinous, convex and entire. Two types of colonies were observed on *Salmonella-Shigella* agar, colorless colonies and colorless colonies with black centers (Supplementary Table I). Regarding biochemical characterization, isolates pfs4, pfs10, pfs16, pfs18, pfs29, pfs34 and pfs37 were catalase positive, oxidase positive, ferment glucose, maltose, sucrose and xylose but without gas production. Isolates pfs13, pfs17, pfs23, pfs30 and pfs38 were found catalase positive, oxidase negative, ferment glucose, maltose, sucrose and xylose but were negative for gas production. Isolates pfs5 and pfs35 were found positive for all biochemical tests except gas production. Isolates pfs6 and pfs36 were found oxidase negative, catalase positive, gave positive reaction for glucose, maltose, sucrose and xylose fermentation without gas production. Isolate pfs24 was found negative for gas production and for glucose fermentation while all other tests were positive. Isolates pfs72 and pfs65 were found catalase positive, oxidase negative and negative for xylose fermentation and gas production. Gram negative isolates pfs41, pfs46, pfs53 and pfs77 were oxidase negative, catalase positive and ferment glucose, maltose, sucrose and xylose with gas production, however isolate pfs103 was negative for sucrose fermentation and isolate pfs107 was found negative for gas production and did not ferment glucose and sucrose (Supplementary Table II).

16S rRNA gene was amplified for thirty-two isolates and was sequenced commercially. Sequence analysis results showed that feed samples were contaminated with different gram positive and gram-negative bacteria (Table III). Phylogenetic tree showed that isolates pfs5, pfs6, pfs10, pfs13, pfs16, pfs17, pfs18, pfs24, pfs29, pfs34, and pfs37 were closely related to *Bacillus* sp., isolate pfs65 showed highest similarity with *Staphylococcus aureus*, isolate pfs41 was related to *Enterobacter cloacae*, isolate pfs77 was closely related to *Escherichia vulneris*, isolates pfs46 and pfs53 were associated with *Ralstonia* sp. Isolates pfs103 and pfs107 were identified as *Salmonella enterica* and *Shigella* sp., respectively (Supplementary Fig. I).

Fungal isolates were obtained on PDA after 3-5 days of incubation at 30°C. Isolate PFF1 gave white colored colony which turned black on maturation; the colony was round and powdery while the back side of colony was pale yellow. Isolate PFF2 had yellowish green circular colony

Table III. Similarity between 16S rRNA gene sequence of isolated isolates with related isolates established on Mega Blast.

S. No.	Isolate	Accession No.	Related isolate	% age similarity
1	pfs2	MK033494	*Bacillus methylotrophicus* NR 116240	95
2	pfs4	MK170135	*Bacillus subtilis* NR 112116	100
3	pfs5	MK170134	*Bacillus tequilensis* NR 104919	99
4	pfs6	MK170133	*Bacillus idriensis* NR 043268	98
5	pfs8	MK170132	*Bacillus humi* NR 025626	96
6	pfs10	MK170131	*Bacillus subtilis* NR 112116	99
7	pfs13	MK170130	*Bacillus haynesii* NR 157609	99
8	pfs14	MK170129	*Bacillus wiedmannii* NR 152692	95
9	pfs15	MK170128	*Bacillus wiedmannii* NR 152692	96
10	pfs16	MK170127	*Bacillus subtilis* NR 112116	99
11	pfs17	MK170126	*Bacillus haynesii* NR 157609	99
12	pfs18	MK170125	*Bacillus subtilis* NR 112116	99
13	pfs23	MK170124	*Bacillus haynesii* NR 157609	99
14	pfs24	MK170123	*Bacillus methylotrophicus* NR 116240	99
15	pfs25	MK170122	*Bacillus nakamurai* NR 151897	97
16	pfs27	MK170121	*Bacillus wiedmannii* NR 152692	96
27	pfs28	MK170120	*Bacillus wiedmannii* NR 152692	98
18	pfs29	MK170119	*Bacillus subtilis* NR 112116	99
19	pfs30	MK170118	*Bacillus haynesii* NR 1576091	97
20	pfs34	MK170117	*Bacillus subtilis* NR 112116	99
21	pfs35	MK170116	*Bacillus tequilensis* NR 104919	99
22	pfs36	MK170115	*Bacillus mojavensis* NR 024693	97
23	pfs37	MK170114	*Bacillus subtilis* NR 112116	99
24	pfs38	MK170113	*Bacillus haynesii* NR 157609	97
25	pfs41	MK170112	*Enterobacter cloacae* NR 118568	100
26	pfs46	MK170111	*Ralstonia syzygii* NR 134150	99
27	pfs53	MK170110	*Ralstonia pickettii* NR 114126	99
28	pfs65	MK170109	*Staphylococcus warneri* NR 025922	100
29	pfs72	MK170108	*Staphylococcus aureus* NR 037007	99
30	pfs77	MK170107	*Escherichia vulnaris* NR 119109	99
31	pfs103	MK170106	*Salmonella enterica* NR 074910	98
32	pfs107	MK170105	*Shigella dysenteriae* NR 026332	97

that turned green on maturation. Isolates PFF1 and PFF2 had septate hyphae and globose spores with blackish brown and green color, respectively. Based on colony morphology and microscopic characteristics (Supplementary Table III), the isolated fungi PFF1 and PFF2 were identified as *Aspergillus niger* and *Aspergillus flavus,* respectively.

DISCUSSION

In the present study, commercially available poultry feed samples, collected from different locations of Pakistan, were analyzed using different media to determine TVC, TCC, total *Salmonella* count, total *Shigella* count and total *Staphylococcus* count. TVC in poultry feed ranged from 6.22-7.07 log CFU/g. Contamination of poultry feed was found in the range of 5.0×10^3 to 1.76×10^6 CFU/g in Ilorin, Nigeria (Sule and Ilori, 2017). Sultana *et al*. (2017) found the TVC of poultry feed (9.5×10^5 CFU/g) in Dhaka region, Bangladesh and in another study in Bangladesh, it was reported as 5.45×10^6 CFU/g (Chowdhury *et al*., 2011). Pavlovic et al. (2019) reported average bacterial contamination (1.95×10^4 CFU/g) in feed for laying hens

from the Serbian market.

EMBA medium gave efficient growth of coliforms. The coliform count was found in the range of 4.30 – 6.19 log CFU/g. Previously reported coliform counts ranged from 0 - 3.0×10^5 CFU/g (Sule and Ilori, 2017), 0 - 6.75×10^4 CFU/g (Chowdhury *et al*., 2011) and 2.68×10^3 - $3.15\text{x}10^4$ CFU/g (Sultana *et al*., 2017). *Staphylococcus* count in the poultry feeds ranged from 5.56-6.89 log CFU/g. *Salmonella* and *Shigella* contamination was in the range of 0-5.53±0.36 log CFU/g and 0-6.54±0.78 log CFU/g, respectively. Chowdhury *et al*. (2011) found the *Salmonella* contamination rate from 0 to 3.05×10^4 CFU/g in poultry feed samples collected from Dhaka, Bangladesh. In contrast, *Salmonella* and *Shigella* were not reported in the poultry samples collected from Nigeria (Sule *et al*., 2017).

According to the Bergey's manual, *Bacillus subtilis* strains have biochemical characteristics similar to those obtained for isolates pfs4, pfs10, pfs16, pfs18, pfs29, pfs34 and pfs37. Biochemical properties of isolates (pfs13, pfs17, pfs23, pfs30 and pfs38) were found similar to the biochemical characteristics reported for *B. haynesi* by Dunlap *et al*. (2017). Biochemical characteristics of isolates (pfs5 and pfs35) matches with *B. tequliensis* (Gatson *et al*., 2006). Isolate pfs8 gave biochemical properties similar to *B. humi* (Heyrman *et al*., 2005). Results of biochemical characteristics of isolates pfs6 and pfs36 were in accordance to the results of Ko *et al*. (2006) and Roberts *et al*. (1994), who reported similar properties for *B. idriensis* and *B. mojavensis*. Biochemical characteristics of isolate pfs24 matches to *B. methylotropicus* (Madhaiyan *et al*., 2010). Isolates (pfs72 and pfs65) gave similar biochemical results as reported by Olugbojo and Ayoola (2015) for *Staphylococcus*. Gram negative isolates (pfs41, pfs46, pfs53, pfs77, pfs107) gave biochemical characteristics similar to the gram-negative isolates (*E. coli*, *Salmonella* sp. and *Shigella* sp.) obtained by Olugbojo and Ayoola (2015). Phylogenetic analysis based on 16S rRNA gene sequences showed that poultry feed samples were contaminated with bacteria belonging to *Salmonella enterica, Staphylococcus aureus, Enterobacter cloacae, Bacillus haynesii, Bacillus subtilis*, *Shigella* sp., *Escherichia* sp. and *Ralstonia* sp.

More contamination was found in chick and grower feed. These results are in accordance with Xiulan *et al*. (2006) who stated that chick feed and grower feed had high contamination due to high protein content. These findings are also in accordance with Yunus *et al*. (2009) who found that diseases were more common in chicks which were fed on commercially available feed as compared to home mixed feed. 100% samples were found positive for *Bacillus* and *Staphylococcus*. The results are in accordance to the results of Roy *et al*. (2017), who reported *Staphylococcus* as the most abundant pathogenic bacteria found in poultry feed samples collected from Dhaka city, Bangladesh. *Staphylococcus* has been found to be associated with bumblefoot disease and arthritis in poultry (Nazia *et al*., 2015). *Salmonella* was found in layer feed samples. This is in accordance to Shirota *et al*. (2000) who stated that layer and broiler feed were more susceptible to *Salmonella*. *Salmonella* is responsible for salmonellosis in poultry. *Salmonella* serotypes have great ability to adapt in number of host environments and diverse routes of transmission so can cause chronic and acute infections in almost all kind of birds and animals (Kwiatek and Kukier, 2008). *Shigella* was found in 8.3% samples. It is reported to be associated with gastrointestinal infections in poultry and humans (Baker and The, 2018). *Enterobacter* is normal inhabitant of gut flora but it can become opportunistic pathogen in immune compressed human and can cause different kinds of respiratory and gastrointestinal infections (Keller *et al*., 1998). *Enterobacter cloacae* was the most common bacterial species identified (54.5%) followed by *Bacillus cereus* (27. 3%) and then *Klebsiella pneumoniae* (18.2%) (Mahami *et al*., 2019). Ngai *et al*. (2021) reported *E. coli* and *Salmonella* in the poultry feed samples from Kenya. Gram-negative bacteria, *Escherichia vulneris* is closely related isolate of *E. coli*. It can cause gastrointestinal, respiratory and urinary infections (Jain *et al*., 2016). *Ralstonia* is soil-inhabiting bacteria and its occurrence in poultry feed samples indicates contamination of feed with soil particles (Ryan *et al*., 2007).

Fungal contamination was comparatively less than bacterial contamination. Only 8.3% samples were positive for fungal contamination. Observed colony and microscopic characteristics of fungal isolates were also supported by the characteristics studied by Pathak and Narulu (2013) and Hedayati *et al*. (2007). Contamination of poultry feed samples with *Aspergillus* had also been reported in Nigeria (Uwaezuoke and Ogbulie, 2010; Anifowose and Bakre, 2021), Bangladesh (Saleemi *et al*., 2020), Poland (Cegielska-Radziejewska *et al*., 2013), Egypt (Laban *et al*., 2014), Kaduna state (Habib *et al*., 2015), Skoto (Aliyu *et al*., 2016), Iraq (Alkhursan *et al*., 2021) and Saudia Arabia (Gherbawy *et al*., 2020). Presence of *Aspergillus* is a great threat to poultry as well as other consumers of poultry. Aflatoxins produced by fungi are found to be associated with sinusitis, keratitis, cutaneous aspergillosis, and systemic infections in individuals having weak immune system. Aflatoxins can also cause cutaneous Aspergillosis in other vertebrates (Hedayati *et al*., 2007). These aflatoxins also contribute to the economic loss of poultry industry as they weaken the immune system of chicks and interfere with the reproductive power of

chickens (Habib *et al*., 2015).

CONCLUSION

Commercialization of the poultry industry has increased risks to human health in the absence of adequate supervision and care taken during the production of poultry feeds. Contamination of poultry feed with microbes not only causes animal health problems but can also pose health risks to humans. Poultry feeds must therefore be introduced into the market after adequate experimental analysis and certification to avoid health risks associated with poultry products.

DECLARATIONS

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Ethical statement

The study was conducted adhering to all the lab safety protocols.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20231129053939

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Microbiological Analysis of Commercially Available Poultry Feed in Pakistan

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Supplementary Table I. Morphological characteristics of isolated bacterial colony.

Isolates	Media	Colony color	Colony shape	Surface	Elevation	Margin
pfs65, pfs72	MSA	Yellow	Circular	Gelatinous	Convex	Entire
pfs4, pfs7, pfs10, pfs16, pfs18, pfs29, pfs34, pfs37	NA	Cream	Circular	Gelatinous	Convex	Undulate
pfs24	NA	Cream	Circular	Gelatinous	Convex	Entire
pfs13, pfs17, pfs23, pfs30	NA	Cream	Circular	Gelatinous	Convex	Entire
pfs14, pfs15, pfs27, pfs28	NA	Cream	Circular	Gelatinous	Convex	Erose
pfs2, pfs25	NA	Orange	Irregular	Gelatinous	Flat	Entire
pfs8	NA	Cream	Circular	Gelatinous	Umbonate	Erose
pfs5, pfs35	NA	Cream	Circular	Gelatinous	Convex	Entire
pfs6, pfs36	NA	Cream	Filamentous	Gelatinous	Flat	Erose
pfs41	MCA	Pink	Circular	Gelatinous	Convex	Entire
pfs46, pfs53	MCA	Light pink	Punctiform	Gelatinous	Convex	Entire
pfs103	SSA	Black centered colorless	Circular	Gelatinous	Flat	Entire
pfs107	SSA	Colorless colonies	Circular	Dry	Flat	Entire
pfs77	EMBA	Black colonies with green sheen	Circular	Gelatinous	Convex	Entire

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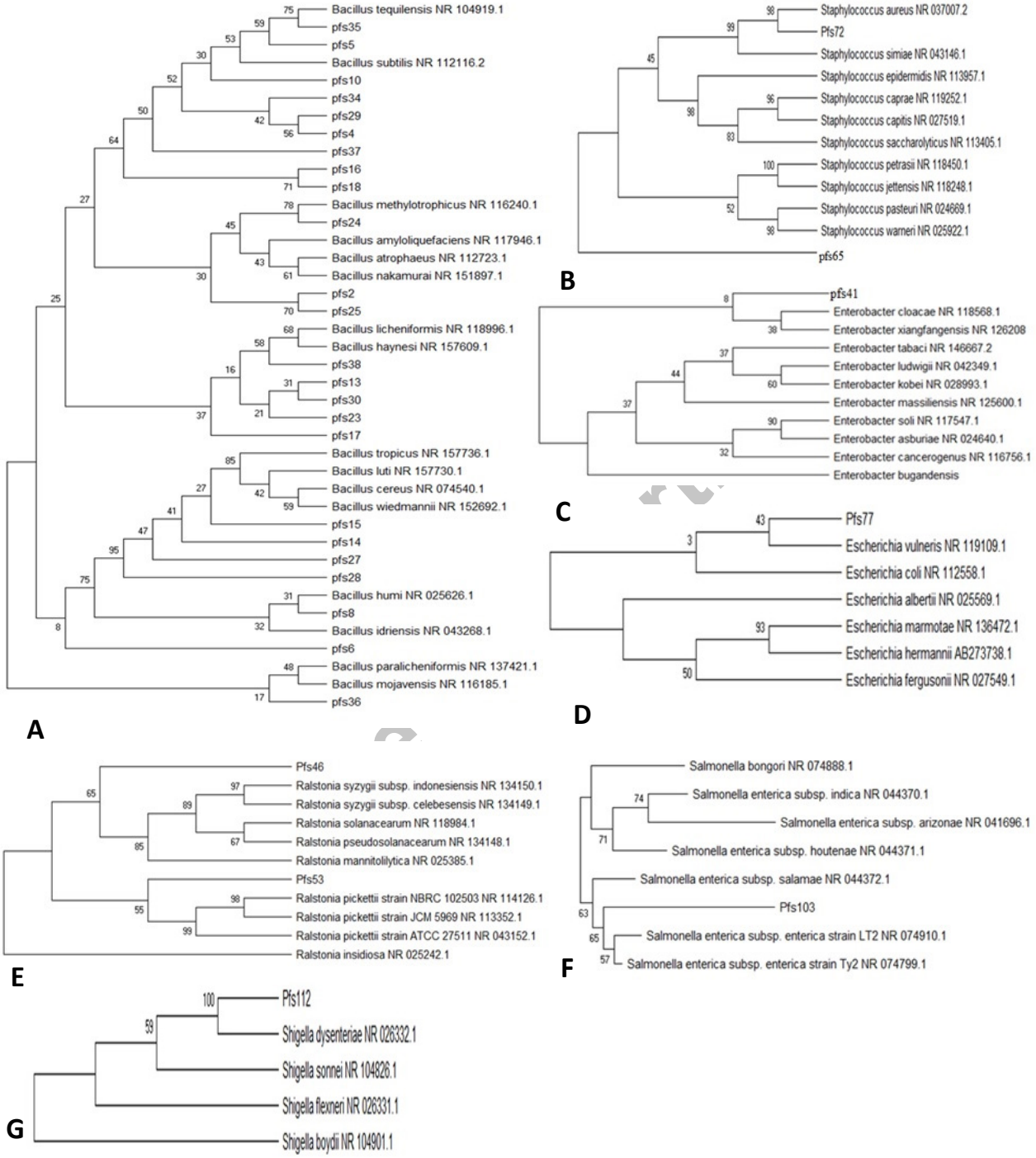
Supplementary Table II. Biochemical characteristics of isolates.

Isolates	Gram stain	Catalase test	Oxidase test	Sugar fermentation test							
				Glucose	Gas production	Maltose	Gas production	Sucrose	Gas production	Xylose	Gas production
pfs65, pfs72	+	+	-	+	-	+	-	+	-	-	-
pfs4, pfs7, pfs10, pfs16, pfs18, pfs29, pfs34, pfs37	+	+	+	+	-	+	-	+	-	+	-
pfs24	+	+	+	-	-	+	-	+	-	+	-
pfs13, pfs17, pfs23, pfs30	+	+	-	+	-	+	-	+	-	+	-
pfs14, pfs15, pfs27, pfs28	+	+	+	+	-	+	-	-	-	+	-
pfs2, pfs25	+	+	+	+	-	+	-	+	-	+	-
pfs8	+	+	+	+	-	-	-	-	-	-	-
pfs5, pfs35	+	+	+	+	-	+	-	+	-	+	-
pfs6, pfs36	+	+	-	+	-	+	-	+	-	+	-
pfs41	-	+	-	+	+	+	+	+	+	+	+
Pfs46, pfs53	+	+	-	+	+	+	+	+	+	+	+
pfs103	-	+	-	+	+	+	+	-	-	+	+
pfs107	-	+	-	-	-	+	-	-	-	+	-

+, positive; -, negative.

Supplementary Table III. Morphological and Microscopic characters of isolated fungi.

Isolate	Morphological characteristics			Microscopic characteristics						
	Colony			Hyphae			Spore			
	Color	shape	Back	Type	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Color	Shape	Identified fungus
PFF1	Black	Spherical and powdery	Pale colored	Septate	300	7	30	2.6	Blackish brown	Globose	*Aspergillus niger*
PFF2	Green	Wooly and circular	Yellow colored	Septate	600	9	38	3.5	Green	Globose	*Aspergillus flavus*



Supplementary Fig. 1. Phylogenetic tree showing evolutionary relationship between isolates and some references *Bacillus strains* (A); *Staphylococcus* (B); *Enterobacter* (C); *Escherichia* (D); *Ralstonia* (E); *Salmonella* (F), and *Shigella* (G) strains.