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Microbiological Evaluation and Genetic Identification of Some Bacterial Species in Soft Cheeses: Implications for Food Safety

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THIS study investigates the microbiological profile of soft cheeses in Egypt, focusing on the identification of *Kocuria flava*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Escherichia coli*. A total of 100 cheese samples were analyzed, and bacterial isolates were characterized using 16S rRNA gene sequencing, with sequences registered in GenBank. Phylogenetic analysis confirmed that the isolates belonged to the genera *Escherichia*, *Bacillus*, and *Kocuria*, closely resembling known strains of *E. coli, B. licheniformis, K. flava,* and *B. subtilis*. Cluster analysis and phylogenetic tree construction revealed genetic similarity with reference strains, with the isolates segregated into two distinct clusters. Microbiological evaluation demonstrated significant variation in bacterial counts (cfu/g) across cheese types. *Salmonella* was detected in higher concentrations in Istanbolly and Tallaga cheeses compared to Kariesh and Feta cheeses. *B. licheniformis* exhibited higher counts in Tallaga, Feta, and Kariesh cheeses than in Istanbolly. *E. coli* was most abundant in Istanbolly cheese, followed by Tallaga and Kariesh, with the lowest levels in Feta cheese. *B. subtilis* showed the highest counts in Feta cheese, while *K. flava* was predominantly found in Istanbolly cheese. These findings highlight the potential presence of harmful microorganisms in soft cheeses, underscoring the importance of stringent hygiene practices during production. Regular microbial assessments and monitoring protocols are essential to detect spoilage bacteria, mitigate health risks, and enhance food safety standards, ensuring the safety of these widely consumed dairy products.

Keywords: Bacterial isolates, Food safety, Microbial contamination.

Introduction

Soft cheese, known for its rich texture and distinctive flavor, is a staple in the daily diets of many consumers worldwide. It holds significant nutritional value, providing proteins, fats, carbohydrates, essential amino acids, fatty acids, minerals, and vitamins (Ibrahim et al., 2015). According to the Egyptian Standards (ES:1867 2005) , soft cheese is a dairy product consumed fresh or stored in brine, produced by the coagulation of milk in the presence of dairy and non-dairy ingredients. However, its high moisture content and low salt levels make it particularly vulnerable to microbial contamination, even under refrigeration (Haddad and Yamani, 2017). While soft cheese offers substantial health benefits, it also poses potential microbiological risks, harboring pathogens such as *Escherichia coli*, *Salmonella*, and *Bacillus* species that can compromise consumer health. Factors such as manufacturing practices, the processing environment, and cross-contamination significantly influence the microbiological quality of cheese (Donnelly, 2018; Possas et al., 2021). Improper handling during production and storage amplifies these risks, highlighting the critical need for stringent adherence to good manufacturing practices (Willis et al., 2022). Additionally, soft

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cheeses produced from unpasteurized milk are frequently linked to outbreaks of foodborne illnesses caused by pathogens like *E. coli* and *Listeria monocytogenes*, particularly in developing countries where regulatory oversight may be limited (Fusco et al., 2020).

Among the microbial contaminants, *Bacillus*, *E. coli*, and *Kocuria* species are of notable concern. *Bacillus* species are resilient, capable of withstanding the harsh conditions encountered during cheese production and storage, with some strains being pathogenic (Zhao et al., 2020; Ewida et al., 2024). *E. coli*, especially pathogenic strains such as *E. coli* O157:H7, is often associated with gastrointestinal infections and may contaminate cheese through poor hygienic practices, insufficient pasteurization, or fecal contamination (Kamal et al., 2017; Alnakip et al., 2023). Although *Kocuria* species are less studied, they have been implicated in cases of cheese spoilage and pose a risk to immunocompromised individuals (Ramos et al., 2021). The presence of pathogens in soft cheese can result in the production of harmful enterotoxins, posing serious health risks and causing significant economic losses (Bastam et al., 2021). Pathogenic microorganisms can invade human tissues and generate toxic metabolic byproducts, such as biogenic amines, while spoilage microorganisms lead to quality deterioration (Atheeb & Maktoof 2023). These risks are exacerbated by the use of raw or improperly pasteurized milk and poor hygiene during processing (Willis et al., 2022). The molecular characterization of microbial hazards in soft cheese is crucial for understanding their prevalence, distribution, and pathogenic mechanisms. Advanced molecular techniques, such as polymerase chain reaction (PCR) and next-generation sequencing (NGS), have enabled the identification of genetic markers associated with virulence and antibiotic resistance in bacterial strains (Atef et al., 2017). Specifically, 16S rRNA gene analysis, widely used for bacterial taxonomy, offers robust insights into the microbial diversity of soft cheese. Amplification and sequencing of 16S rRNA gene fragments using universal primers allow for precise identification and classification of bacterial species based on highly conserved genetic regions.

This study aims to bridge a critical knowledge gap in soft cheese safety by combining traditional microbiological methods with advanced molecular tools to identify and classify foodborne pathogens, including lesser-known species such as *Kocuria flava* and *Bacillus licheniformis*. By analyzing market-available soft cheese, the study provides a comprehensive assessment of contamination rates, highlighting the alarming prevalence of microbiologically unsafe products. The findings offer practical recommendations to enhance food safety practices and inform policymakers, manufacturers, and public health officials, thereby contributing to the establishment of stricter safety standards for cheese production and distribution.

Materials and Methods

Collection of samples

A total of 100 soft cheese samples, comprising four types (25 samples each of Tallaga, Feta, Istanbolly, and Kariesh cheeses), were collected from various markets. These cheese varieties were selected due to their widespread consumption and popularity in the region, representing a significant portion of the soft cheese market. Tallaga, Feta, and Istanbolly cheeses are staples in households and widely used in food services, while Kariesh cheese is particularly valued for its traditional roots and perceived health benefits. To ensure representativeness, samples were procured from a diverse range of markets spanning urban, peri-urban, and rural areas. This approach was designed to capture variations in production practices, storage conditions, and potential contamination risks, thereby providing a comprehensive view of the types of cheeses available to consumers. Upon collection, all samples were promptly refrigerated and stored under appropriate conditions to preserve their integrity for subsequent microbiological and molecular analyses.

Microbiological evaluation of the examined soft cheese

All microbiological media used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Oxoid (Basingstoke, UK). Microbiological examinations were conducted in accordance with the standard protocols outlined by the American Public Health Association (APHA, 2004) and the International Organization for Standardization (ISO, 2013). These media were chosen based on their validated efficacy in isolating *E.coli, Bacillus*, and *Kocuria* species, as demonstrated in previous studies. Approximately 10 g of each cheese sample were aseptically removed using a sterile spoon and homogenized with 90 mL of 2% sodium citrate solution under sterile conditions. Serial tenfold dilution (up to 10^6) was prepared to ensure the accurate enumeration

of microorganisms. Subsequently, 1 mL from each dilution was inoculated onto the appropriate selective media for the isolation and enumeration of the target microorganisms. The diluted sample was inoculated onto selected medium by surface spreading or pours plating to facilitate the growth of colonies, and then the plates were incubated at desired temperature for a desired time allowing for colony development and subsequent isolation of bacterial strains. When required, the 1 mL diluted sample was inoculated into sterile liquid broth medium to enrich bacterial growth. The inoculated broth was incubated under shaking or static conditions at desired temperature for desired time to achieve optimal bacterial proliferation.

Detection and isolation of microorganisms

Coliforms were enumerated and isolated using MacConkey agar, a selective and differential medium. The inoculated plates were incubated at 37 °C for 24 h to promote the growth of coliform colonies. Detection of *Salmonella* spp. was performed using Salmonella-Shigella (SS) agar, a selective medium designed for the isolation of *Salmonella* and *Shigella*. The plates were inoculated and incubated at 37 °C for 24 h. Characteristic *Salmonella* colonies appeared small, translucent, and either lacked pigmentation or exhibited black centers due to hydrogen sulfide production. To detect and enumerate *Bacillus* spp., reinforced clostridial agar was employed, with plates incubated at 37 °C for 24 h. This medium supports the growth of spore-forming bacteria, including *Bacillus* species, under aerobic conditions. Since a specific selective medium for isolating *Kocuria* spp. is not available, mannitol salt agar (MSA) was used as an alternative. MSA is selective for Gram-positive, salt-tolerant microorganisms, including members of the genus *Kocuria*. The high salt concentration (7.5% NaCl) inhibits the growth of non-halotolerant organisms, thereby facilitating the isolation of target species. Plates were incubated at 35 °C for 24–48 h, as this temperature aligns with the optimal growth conditions for *Kocuria* spp., which thrive in mesophilic environments. Four representative isolates (*K. flava, B. licheniformis, B. subtilis,* and *E. coli*) were selected for 16S rRNA gene sequencing. These isolates were chosen based on their potential relevance to food safety concerns, as indicated by their prevalence in soft cheese samples and their known association with spoilage or pathogenicity in dairy products. Criteria were used for selection is the frequency of isolation, where isolates that appeared consistently across multiple cheese samples or exhibited high prevalence in specific cheese types were prioritized. Also, Isolates belonging to genera or species known for their pathogenicity, spoilage potential, or industrial relevance were chosen. In addition, unique or distinct phenotypic traits observed during culturing, such as colony morphology, pigmentation, or growth patterns, informed the selection. Moreover, Isolates with unexpected or rare traits compared to known patterns in cheese microbiology were included to explore potential novel findings.

Molecular characterization of selected bacterial isolates

Isolation of the genome and amplification of the 16S rRNA gene

The Quick-DNA™ Fungal/Bacterial Miniprep Kit was used to extract DNA from bacterial isolates. The quality and purity of the extracted DNA were assessed by measuring the absorbance ratio at 260/280 nm using a spectrophotometer, providing an indication of protein contamination. DNA concentration was determined to ensure sufficient quantities for downstream analyses, ensuring that the extracted DNA was of high quality and purity for accurate molecular characterization (Cheng et al., 2010). The 16S rRNA gene, rather than the ITS region, was amplified using universal primers (Forward primer: 5′-GAG AGT TTG ATC CTG GCT GGC TCA G-3′ and Reverse primer: 5′-AAG GAG GTG ATC CAG CCG CA-3′) (Cheng et al., 2010). These primers were designed to amplify the nearly full-length 16S rRNA gene (~1500 bp), which includes conserved and variable regions (V1-V9). Notably, the primers utilized align with the widely recognized 8F/27F – 1492R set. The amplification of the full 16S rRNA gene enables comprehensive phylogenetic analysis and species identification. PCR reactions were performed in a total volume of 50 µl, containing 100 ng of template DNA, 0.2 µM of each primer, 200 µM of each dNTP, 2.5 U of Taq polymerase enzyme, 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), and nuclease-free water. PCR amplification was carried out using a thermal cycler 2720 (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. The amplified PCR products were purified using the Wizard RSV Gel and PCR Clean-Up System (Promega catalog number A928) following

the manufacturer's instructions to remove unincorporated primers and other impurities. The purified PCR products were sequenced using the Solgent Sequencing Service in Korea. The 16S rRNA sequences obtained from the four bacterial isolates—E. coli, B. licheniformis, K. flava, and B. subtilis (D1, D2, D3, and D5)—were submitted to GenBank and assigned accession numbers PP563664, PP563660, PP559501, and PP562370, respectively. For clarity, Salmonella was not isolated due to technical issues.

Sequence analysis of the 16S rRNA gene and phylogenetic analysis

The purified PCR products were sequenced using the Sanger method at Solgent Sequencing Service, Korea. The resulting 16S rRNA gene sequences from the four bacterial isolates were submitted to GenBank and assigned accession numbers. To determine their identities, the nucleotide sequences were compared to those of *Escherichia coli*, *Bacillus licheniformis*, *Kocuria flava*, and *Bacillus subtilis* using the BLAST tool available at the NCBI database (http://www.ncbi.nlm.nih.gov/Blast), specifically the 16S ribosomal RNA (bacteria and archaea type strains) database (Benson et al*.,* 1999). Sequence alignments with reference sequences from the NCBI database were conducted using MEGA X software, ensuring accurate comparisons (Kumar *et al.,* 2018). A phylogenetic dendrogram was subsequently constructed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm implemented in MEGA X, providing insights into the evolutionary relationships among the isolates and their closest relatives. The 16S rRNA gene sequences of the bacterial isolates ranged from 1300 to 1500 base pairs, depending on the quality and completeness of the PCR amplification and sequencing results. For the identification process, the sequences were matched against known bacterial strains in a sequence database. A 100% similarity was achieved when the query coverage was ≥99%, ensuring high confidence in the taxonomic identification. The *NCBI GenBank* database was used for the sequence comparison and matching process. This database was selected due to its extensive repository of bacterial 16S rRNA sequences, which provided robust reference data for accurate species-level identification.

Results and Discussion

Detection of isolate microorganisms in cheese

Table 1 presents the log counts (cfu/g) of *Salmonella* spp. detected in the examined soft cheese samples. The results revealed that all types of soft cheese analyzed were contaminated with *Salmonella*, with notably high counts across the samples. Among the tested varieties, Istanbolly cheese exhibited the highest mean *Salmonella* count (1.91 log cfu/g), followed by Tallaga cheese (1.87 log cfu/g) and Kariesh cheese (1.50 log cfu/g). Feta cheese had the lowest mean *Salmonella* count (0.99 log cfu/g). Regarding the prevalence of *Salmonella* contamination, 80% of the Tallaga cheese samples tested positive for the pathogen, making it the most affected type in terms of frequency. Similarly, 72% of the Istanbolly cheese samples were positive for *Salmonella*, followed by 64% of Kariesh cheese samples. Feta cheese showed the lowest prevalence, with 48% of the collected samples testing positive for *Salmonella*. These findings underscore the widespread presence of *Salmonella* in soft cheese, highlighting the urgent need for stringent safety measures in production, storage, and handling practices to mitigate contamination and protect public health. The higher count of *Salmonella* present in Tallaga cheese might be due to its lower salt content and higher moisture, which provide a more favorable environment for bacterial growth. In contrast, the lower count in Feta cheese can be attributed to its acidic conditions and higher salt content, both of which inhibit the growth of *Salmonella*. Similar observations have been reported in previous studies, where low salt concentrations and high moisture levels were associated with increased microbial contamination in soft cheeses (D'amico, 2014; Nazem et al*.,* 2020; Lobacz & Zulewska 2021). Furthermore, the role of acidity in suppressing *Salmonella* growth has been well-documented in fermented dairy products (Elafify et al., 2022). These findings highlight the importance of salt and pH as critical factors influencing bacterial contamination in cheese. These results were in line with those of (Ibrahim et al., 2015) who detected *Salmonella* in Domiati and Kariesh cheeses collected from the Cairo governorate. In addition, (Ayaka et al., 2022) and (Ahmed et al., 2022) detected *Salmonella* in collected soft cheese samples. The high prevalence of *Salmonella* detected in the cheese samples could be attributed to environmental contamination during production, handling, or storage. In traditional cheese-making processes, inadequate hygiene practices, such as unclean equipment, contaminated water sources, or improper handling by workers, may introduce or amplify *Salmonella* contamination. Additionally,

environmental factors such as proximity to livestock or poorly maintained production facilities can increase the risk of contamination. Specific handling practices, including inadequate refrigeration and prolonged exposure to ambient temperatures during transportation or sale, may further promote the survival and proliferation of *Salmonella*. Similar findings have been reported in previous studies, where the prevalence of Salmonella was linked to unsanitary conditions in cheese production environments (Miller et al., 2019; Smith et al., 2020). Addressing these issues requires stricter enforcement of hygiene protocols, training for dairy workers on proper handling practices, and improved environmental sanitation to reduce the risk of contamination in soft cheeses (Codex, 2020). Understanding and mitigating these risks is critical to ensuring the safety of cheese products (Hassan & Gomaa, 2016). The Egyptian standards (ES) specify that soft cheese must be free from *Salmonella*. All 25 Feta cheese samples were tested against ES requirements, and 13 samples (representing 52% of the total) failed to comply with the standard due to the presence of *Salmonella.* Nine out of twenty-five Kariesh cheese samples complied with ES, representing 36% of the collected samples. However, only seven out of twenty-five Istanbolly cheese samples complied with ES, representing 28% of the collected samples, and five out of twenty-five Tallaga cheese samples complied with ES, representing 20% of the collected samples.

The results in Table 2 show the log counts (cfu/g) of *B. licheniformis* detected in different soft cheese samples. All 25 samples collected from Feta, Istanbolly, and Kariesh cheeses were positive for *B. licheniformis*, representing a 100% contamination rate. The mean counts were highest in Kariesh cheese (2.76 log cfu/g), followed by Feta cheese (2.61 log cfu/g) and Tallaga cheese (2.52 log cfu/g). The lowest mean count was observed in Istanbolly cheese (1.98 log cfu/g). For Tallaga cheese, 21 out of 25 samples (84%) were positive for *B. licheniformis*. Regarding compliance with the Egyptian standards (ES), which mandate that soft cheese, must be free from *B. licheniformis,* none of the Istanbolly, Feta, or Kariesh cheese samples complied. Only four of the 25 Tallaga cheese samples met the ES standards, representing a compliance rate of 16%. *B. licheniformis* may reach milk from contaminated animal udder, milking equipment and the environment. It can be found as spores that can survive pasteurization and multiply in milk, producing toxins with health risks (Lindström et al., 2010). *B. licheniformis* is a spore-forming bacterium known to survive pasteurization processes and thrive in environments conducive to cheese manufacturing. While not typically pathogenic, it poses risks in dairy products, including cheese, through spoilage and potential toxin production (Gopal et al., 2015). The bacterium can produce proteolytic and lipolytic enzymes, leading to undesirable flavors, textures, and off-odors in cheese. It is also associated with spoilage defects in cheese, such as off-flavors and gas production. Some strains can decarboxylate amino acids, leading to the formation of biogenic amines like histamine and tyramine, which can cause toxic reactions in humans if consumed in high concentrations (Yeak et al., 2022). The presence of *B. licheniformis* in cheese can compromise product safety, reduce shelf life, and negatively impact consumer perception. To mitigate these risks, preventive measures such as strict hygiene protocols, regular monitoring for spore-forming bacteria, and optimizing thermal processing are essential (Falih et al., 2024).

TABLE 1. Statistical analysis of Salmonella spp. counts (cfu/g) in the examined soft cheese samples.

Product	No of examined samples	Positive samples	Mean±SD	ES (2005)	Samples comply with ES	Compliance %	p-Value
Tallaga cheese	25	20	1.87±1.08	Free	5	20%	<0.05
Feta cheese	25	12	0.99±1.10	Free	13	52%	<0.05
Istanbolly cheese	25	18	1.91±1.23	Free	7	28%	<0.05
Karish cheese	25	16	1.50±1.19	Free	9	36%	<0.05

P-values indicate statistically significant differences in contamination levels among cheese types.

TABLE 2. Statistical analysis of the *B. licheniformis* count (cfu/g) of the examined soft cheese samples.

Product	No of examined samples	Positive samples	Mean±SD	ES (2005)	Samples comply with ES	Compliance %	p-Value
Tallaga cheese	25	21	2.52±1.15	Free	4	16	<0.05
Feta cheese	25	25	2.61±0.25	Free	0	0	<0.05
Istanbolly cheese	25	25	1.98±0.27	Free	0	0	<0.05
Kariesh cheese	25	25	2.76±0.28	Free	0	0	<0.05

.P-values indicate statistically significant differences in contamination levels among cheese types

Table 3 shows the log counts (cfu/g) of *E. coli* detected in different soft cheese samples. All soft cheese samples contained coliform bacteria, with *E. coli* counts exceeding the Egyptian standards (ES) limits in all cases. Among the examined samples, Istanbolly cheese had the highest mean *E. coli* count (2.96 log cfu/g), followed by Tallaga cheese (2.59 log cfu/g) and Kariesh cheese (2.27 log cfu/g). The lowest count was observed in Feta cheese (1.59 log cfu/g). Regarding contamination prevalence, all 25 samples (100%) of Istanbolly cheese were positive for *E. coli.* In Tallaga cheese, 24 out of 25 samples (96%) tested positive, followed by Kariesh cheese with 21 out of 25 samples (84%) testing positive. In contrast, only 14 out of 25 samples (56%) of Feta cheese were positive for *E. coli*. Similar results were reported by (Awad, 2016), who detected coliform bacteria in Kariesh cheese samples, and (Hassan & Gomaa, 2016)**,** who investigated the microbiological hazards of soft cheese marketed in Giza and Cairo Governorates. In addition, (Lotfy et al., 2018) detected higher coliform counts in some soft cheese samples collected from Cairo, and (Mohamed et al*.,* 2019) reported higher coliform counts in some soft cheeses sold in different governorates in Egypt. Moreover, (Ayaka et al., 2022) reported similar results. The presence of gluconic acid in Feta cheese may explain the lower *E. coli* counts detected in the analyzed samples. According to Egyptian standards (ES), soft cheese should contain no more than 10 *E. coli* cells per gram of cheese. Approximately 44% of the collected Feta cheese samples met the ES criteria, with eleven out of the twenty-five samples testing positive for *E. coli*. In contrast, only 16% of the Kariesh cheese samples (four out of twenty-five) complied with ES. Tallaga cheese showed even poorer compliance, with only one out of twenty-five samples (4%) meeting the standard. None of the Istanbolly cheese samples met the ES criteria, reflecting a concerning level of contamination in this cheese type. The higher coliform counts observed across the soft cheese samples indicate fecal contamination and the potential presence of enteric pathogens. Such contamination is often attributed to unhygienic practices during cheese production, including improper pasteurization and the use of contaminated ingredients (El-Kosi, 2001; de Oliveira et al., 2017). Additionally, contamination may occur during packaging and storage due to substandard hygienic conditions in these environments, as well as inadequate sanitation of workers' hands. Preventing coliform contamination in soft cheese requires strict adherence to hygienic practices throughout the production process. Key measures include applying sufficient heat treatment to cheese milk, ensuring the cleanliness of raw materials, and implementing proper handling and sanitization protocols for all equipment and surfaces in contact with cheese. Adhering to these practices is essential to mitigate the risk of contamination with coliform groups and ensure complain standards (Kousta et al., 2010).

TABLE 3. Statistical analysis of the log E. coli count (cfu/g) of the examined soft cheese samples.

Product	No of examined samples	Positive samples	Mean ±SD	ES (2005)	Samples comply with ES	Compliance %	p-Value
Tallaga cheese	25	24	2.59±0.68	Not more than 10 cell/g	1	4%	<0.05
Feta cheese	25	14	1.59±1.46	Not more than 10 cell/g	11	44%	<0.05
Istanbolly cheese	25	25	2.96±0.27	Not more than 10 cell/g	0	0%	<0.05
Kariesh cheese	25	21	2.27±1.08	Not more than 10 cell/g	4	16%	<0.05

p-values indicate statistically significant differences in contamination levels among cheese types.ce with food

Table 4 presents the log counts (cfu/g) of *Bacillus subtilis* detected in different soft cheese samples. *B. subtilis* was identified in all examined cheese types, with relatively high counts. Istanbolly cheese showed the highest mean *B. subtilis* count (2.69 log cfu/g), followed by Feta cheese (2.40 log cfu/g) and Kariesh cheese (2.08 log cfu/g). The lowest mean count was observed in Tallaga cheese (2.04 log cfu/g). In terms of prevalence, *B. subtilis* was detected in 100% of the Istanbolly cheese samples. Feta cheese had a positivity rate of 88% (22 out of 25 samples), while 84% (21 out of 25 samples) of Kariesh cheese samples tested positive. For Tallaga cheese, *B. subtilis* was present in 80% of the collected samples. These results are in line with those of (Ibrahim et al., 2015; Hassan & Gomaa 2016; Abo El-Makarem et al., 2017; Ahmed et al., 2018; Ayaka et al., 2022), who reported the presence of *Bacillus* in collected soft cheese samples with counts higher than the ES limits. These findings highlight poor manufacturing practices during cheese processing, which increase the risk of contamination and pose a threat to consumer health. Factors such as the use of low-quality raw milk, unclean utensils, and inadequate handling practices likely contribute to the presence of Bacillus species (Ahmed et al., 2018). According to Egyptian standards (ES), soft cheese must be free from *B. subtilis*. However, compliance rates varied among the cheese types. For Tallaga cheese, 20% of the collected samples (5 out of 25) met the ES requirements. In Kariesh cheese, 16% of the samples (4 out of 25) were free of *B. subtilis*, while only 12% (3 out of 25) complied fully with ES limits. None of the Istanbolly cheese samples met the ES standards, as *B. subtilis* was detected in all of them.

Table 5 presents the log counts (cfu/g) of *K. flava* detected in different soft cheese samples. *K. flava* was identified in all examined cheese types, with Istanbolly cheese showing the highest mean count (2.50 log cfu/g), followed by Feta cheese (1.53 log cfu/g), Kariesh cheese (1.36 log cfu/g), and Tallaga cheese, which had the lowest mean count (0.48 log cfu/g). Soft cheese provides a favorable environment for the growth of *K. flava* and other microorganisms, primarily due to contamination from raw milk or cross-contamination during cheese production. In terms of prevalence, *K. flava* was detected in 24 out of 25 Istanbolly cheese samples (96%). For Feta cheese, 18 out of 25 samples (72%) tested positive, while 16 out of 25 Kariesh cheese samples (64%) contained *K. flava*. In contrast, only 6 out of 25 Tallaga cheese samples (24%) were positive for the presence of *K. flava*. Our results agree with those of (Hassan & Gomaa, 2016; Lotfy et al., 2018; Ayaka et al., 2022), which reflect the poor sanitary conditions followed during the production of such cheese types.

According to Egyptian standards (ES), soft cheese must be free of *K. flava*. Compliance rates varied among the cheese types. For Tallaga cheese, 76% of the collected samples (19 out of 25) complied with the ES requirements, as they were free from *K. flava*. In Kariesh cheese, 36% of the samples (9 out of 25) met the ES standards. Similarly, 28% of the Feta cheese samples (7 out of

25) were free of *K. flava*. However, only 4% of the Istanbolly cheese samples (1 out of 25) complied with ES, with *K. flava* being detected in nearly all the samples. Like other *Kocuria* species, *K. flava* can contribute to spoilage of dairy products by altering their sensory properties through enzymatic activity. This may result in undesirable flavors, textures, or odors in cheese (Ayaka et al., 2022). Although many *Kocuria* species are not primary producers of biogenic amines, specific strains could pose a risk under certain conditions by decarboxylating amino acids present in cheese. This could lead to the accumulation of compounds such as histamine and tyramine, which may pose health risks if consumed in significant amounts. *K. flava*, like other environmental bacteria, can colonize processing surfaces and equipment in cheese production facilities, potentially leading to contamination and quality control challenges (Stobnicka-Kupiec et al., 2019). These risks highlight the importance of stringent monitoring and hygiene practices in dairy processing to minimize the impact of *K. flava* and other spoilage-related bacteria on cheese quality and safety. In addition, the presence of *Kocuria* in cheese samples poses significant health risks due to its ability to produce thermostable enterotoxins, which can cause food poisoning when its count reaches 10^6 cfu/g (Youn & Seo, 2022). This underscores the critical importance of pasteurizing milk during soft cheese production to eliminate such harmful microorganisms and prevent potential health hazards.

TABLE 4. Statistical *analysis of the log B. subtilis* count (cfu/g) of the examined soft cheese samples.

Product	No of examined samples	Positive samples	Mean±SD	ES (2005)	Samples comply with ES	Compliance %	p-Value
Tallaga cheese	25	20	2.04±1.65	Free	5	20	<0.05
Feta cheese	25	22	2.40±0.95	Free	3	12	<0.05
Istanbolly cheese	25	25	2.69±0.41	Free	0	0	<0.05
Kariesh cheese	25	21	2.08±0.95	Free	4	16	<0.05

.P-values indicate statistically significant differences in contamination levels among cheese types

TABLE 5. Statistical analysis of log *k. flava* count (cfu/g) in the examined soft cheese samples.

Product	No of examined samples	Positive samples	Mean±SD	ES (2005)	Samples comply with ES	Compliance %	p-Value
Tallaga cheese	25	6	0.48±0.87	Free	19	76	<0.05
Feta cheese	25	18	1.53±0.98	Free	7	28	<0.05
Istanbolly cheese	25	24	2.50±0.74	Free	1	4	<0.05
Kariesh cheese	25	16	1.36±1.05	Free	9	36	<0.05

.P-values indicate statistically significant differences in contamination levels among cheese types

Figure 1 and 2 summarizes the log counts (cfu/g) and occurrence of various bacterial strains detected in the collected soft cheese samples. *Salmonella* was found in higher concentrations in Istanbolly and Tallaga cheese samples compared to Kariesh and Feta cheeses. *B.licheniformis* showed greater counts in Tallaga, Feta, and Kariesh cheese samples than in Istanbolly cheese. *E.coli* was most abundant in Istanbolly cheese, followed by Tallaga and Kariesh cheeses, with the lowest counts observed in Feta cheese. *B. subtilis* was detected at the highest levels in Feta cheese, while *K. flava* was more prevalent in Istanbolly cheese compared to the other types. The microbiological analysis of the soft cheese samples revealed insufficient sanitary conditions during processing and handling. Soft cheese provides a favorable environment for the growth of spoilage microorganisms, emphasizing the need for improved manufacturing practices. To ensure microbiological safety, good manufacturing practices (GMP) should be strictly implemented during production. Key measures include using high-quality milk, applying proper heat treatment, maintaining hygienic practices, regularly sanitizing equipment, and ensuring careful handling throughout the processing chain. Additionally, implementing a continuous monitoring and inspection system is critical to identifying critical control points in the production process. Such systems help prevent microbial contamination and ensure compliance with safety standards (Aslani et al., 2024).

Molecular characterization and phylogenetic analyses of the rDNA sequences

The 16S rRNA gene, known for its high conservation, is beneficial for distinguishing between closely related bacterial species, making it ideal for identifying pathogens in food products. The results of the phylogenetic analysis demonstrated that the isolates belonged to three distinct genera, namely, *Escherichia, Bacillus, and Kocuria*. By comparing the amplified PCR products to sequences stored in GenBank (http://www.ncbi.nlm.nih.gov), the isolates were identified and found to closely resemble known strains of *E. coli*, *B. licheniformis*, *K. flava* and *B. subtilis*, as depicted in Table 6, 7, 8, 9 and 10. The similarity index employed in the cluster analysis also confirmed the affinity between the isolates and known strains.

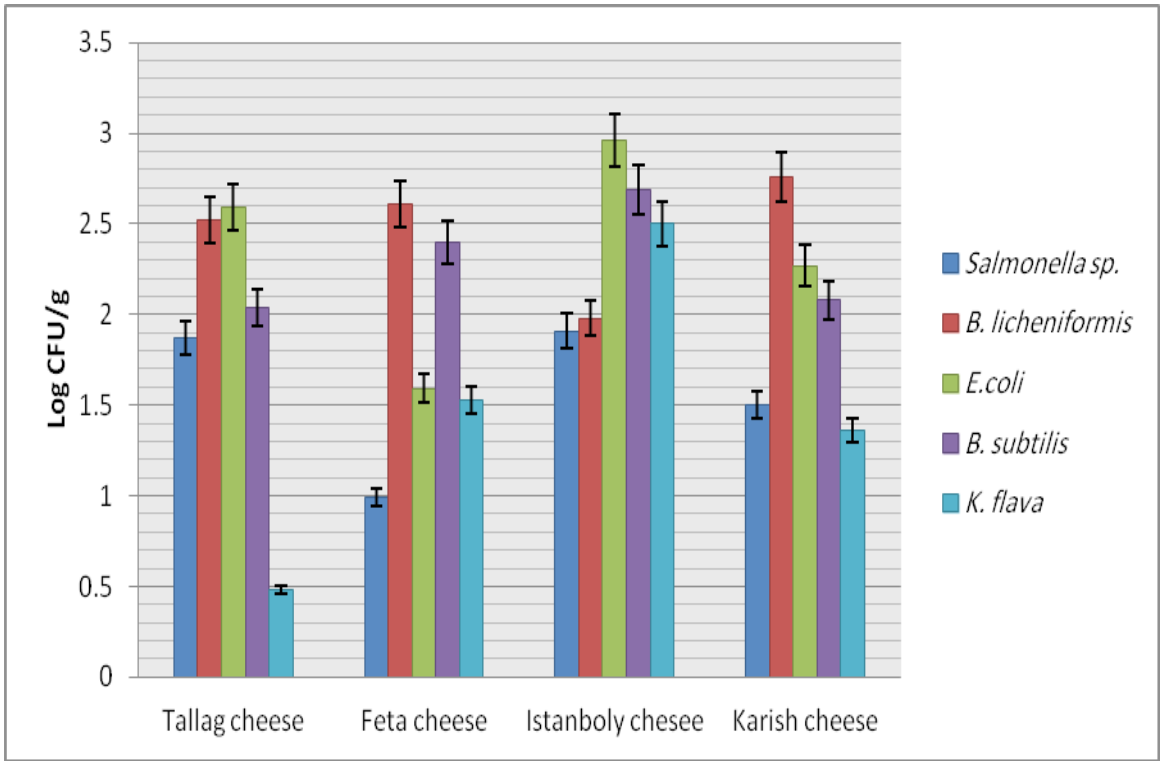

Fig. 1. Log cfu/g counts of different spoilage bacteria detected in soft cheese samples.

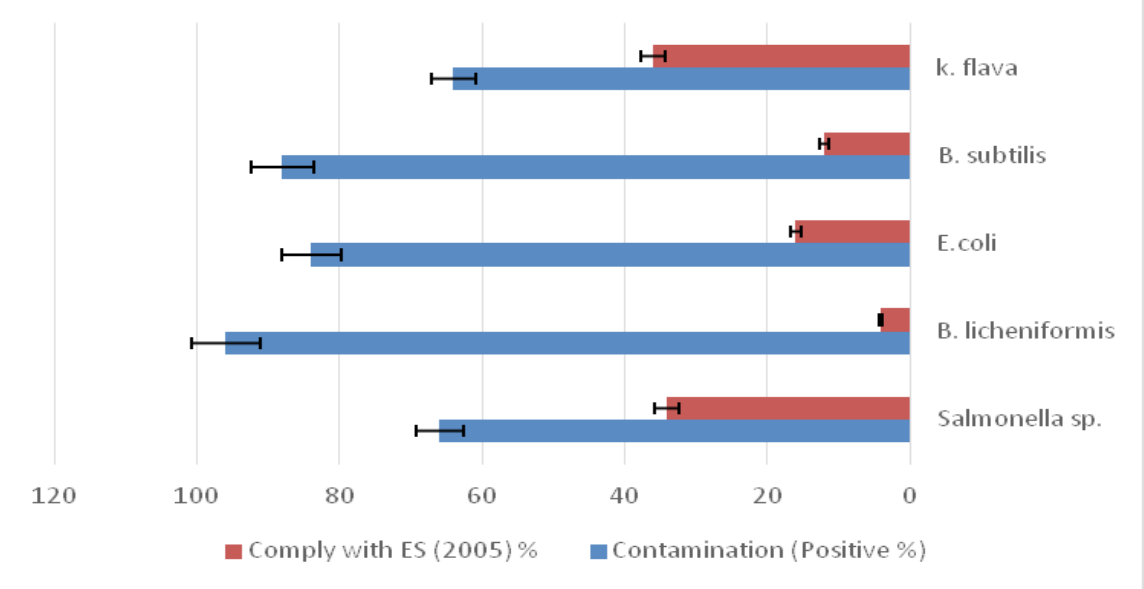

Fig 2. The occurrence of spoilage contamination in collected soft cheese samples.

TABLE 6. Identification of bacterial isolates on the basis of 16S rRNA gene sequence similarity.

Isolate code	Accession no.	Strain
D1	PP563664	*Escherichia coli*
D2	PP563660	*Bacillus licheniformis*
D3	PP559501	*Kocuria flava*
D5	PP562370	*Bacillus subtilis*

TABLE 7. Genetic similarity percentages of 5 strains of *E.coli* and isolate D1 (accession number PP563664).

	1	2	3	4	5	6
1	100	100	97.02	97.14	97.02	96.55
2		100	96.80	96.91	96.80	96.45
3			100	98.50	98.85	91.97
4				100	97.10	92.30
5					100	95.54
6						100

1: |PP563664|*E. coli*-D1, 2: |MT180599|*E. coli,* 3: |PP463722| *E. coli,*
4: |JQ404467| *E. coli*, 5: |GU594294| *E. coli* and 6: |OQ719882| *E. coli*

TABLE 8. Genetic similarity percentages of ٥ strains of *B. licheniformis* and isolate D2 (accession number PP563660).

	1	2	3	4	5	6
1	100	100	100	99.52	98.41	98.81
2		100	100	100	99.93	98.94
3			100	100	99.93	98.94
4				100	99.93	98.94
5					100	100
6						100

PP563660|B.licheniformis-D2, 2: |MK583945| B. licheniformis, 3: |KY063593| B. licheniformis, 4: |MW725566| B.| :1 licheniformis, 5: |MT184857| B. licheniformis and 6: |MG428975| B. licheniformis

TABLE 9. Genetic similarity percentages of 5 strains of *K. flava* and isolate D3 (accession number PP559501).

	1	2	3	4	5	6
1	100	100	98.72	99.17	98.72	98.72
2		100	98.86	99.26	98.86	99.66
3			100	99.29	99.26	99.26
4				100	99.66	99.66
5					100	99.60
6						100

1: |PP559501|*K.flava*-D3, 2: |MG733647| *K.flava*, 3: |MG892795| *K.flava*,
4: |LT223584| *K.flava*, 5: |MG705564| *K.flava* and 6: |MG733641| *K.flava*

TABLE 10. Genetic similarity percentages of ٥ strains of *B. subtilis* and isolate D5 (accession number PP562370).

	1	2	3	4	5	6
1	**100**	100	100	100	100	100
2		**100**	100	100	99.13	100
3			**100**	99.30	100	100
4				**100**	100	100
5					**100**	100
6						**100**

1: |PP562370|*B. subtilis*–D5, 2: |MT498779| *B. subtilis*, 3: |LC543400| *B. subtilis*,
4: |MN865800| *B. subtilis*, 5: |MH198042| *B. subtilis* and 6: |ON063339| *B. subtilis*

The phylogenetic analysis revealed the segregation of the isolates into two distinct clusters, each corresponding to a specific genus and showing significant similarity to known bacterial strains, as depicted in Fig. 3. The clustering of isolates into two distinct groups in the phylogenetic tree is significant as it reflects the evolutionary divergence between the bacterial genera represented in the samples. This segregation not only supports the taxonomic classification of the isolates but also provides valuable insights into their ecological roles and functional attributes.

The observed clustering suggests that these bacterial groups may have adapted to distinct environmental niches or functional roles within their ecosystem. For instance, one genus may be associated with specific metabolic pathways or environmental interactions, while the other may exhibit traits critical to survival under different conditions (Konstantinidis & Tiedje, 2005). Furthermore, the high similarity of the isolates to known strains highlights the potential conservation of key genetic features, which could be linked to shared evolutionary pressures or functional importance Schloss (Schloss & Handelsman, 2004). This study provides new insights into the diversity of bacterial species in the collected samples while emphasizing the utility of 16S rRNA gene sequencing for precise bacterial identification. The cluster analysis highlighted a high degree of similarity between the bacterial isolates and previously identified species. Notably; Isolate D1 (accession number |PP563664|) exhibited a 100% similarity with the *E. coli* strain |MT180599|. Isolate D2 (accession number |PP563660|) showed a 100% similarity with *B. licheniformis* strains |MK583945| and |KY063593|. Isolate D3 (accession number |PP559501|) demonstrated a 100% similarity with *K. flava* strains |MG733647|. Isolate D5 (accession number |PP562370|) matched with *B. subtilis* strains |MT498779|, |LC543400|, |MN865800|, |MH198042|, and |ON063339|, all with a 100% similarity index. In conclusion, this study underscores the close phylogenetic relationships between the bacterial isolates and these specific species, highlighting the effectiveness of molecular techniques in bacterial characterization.

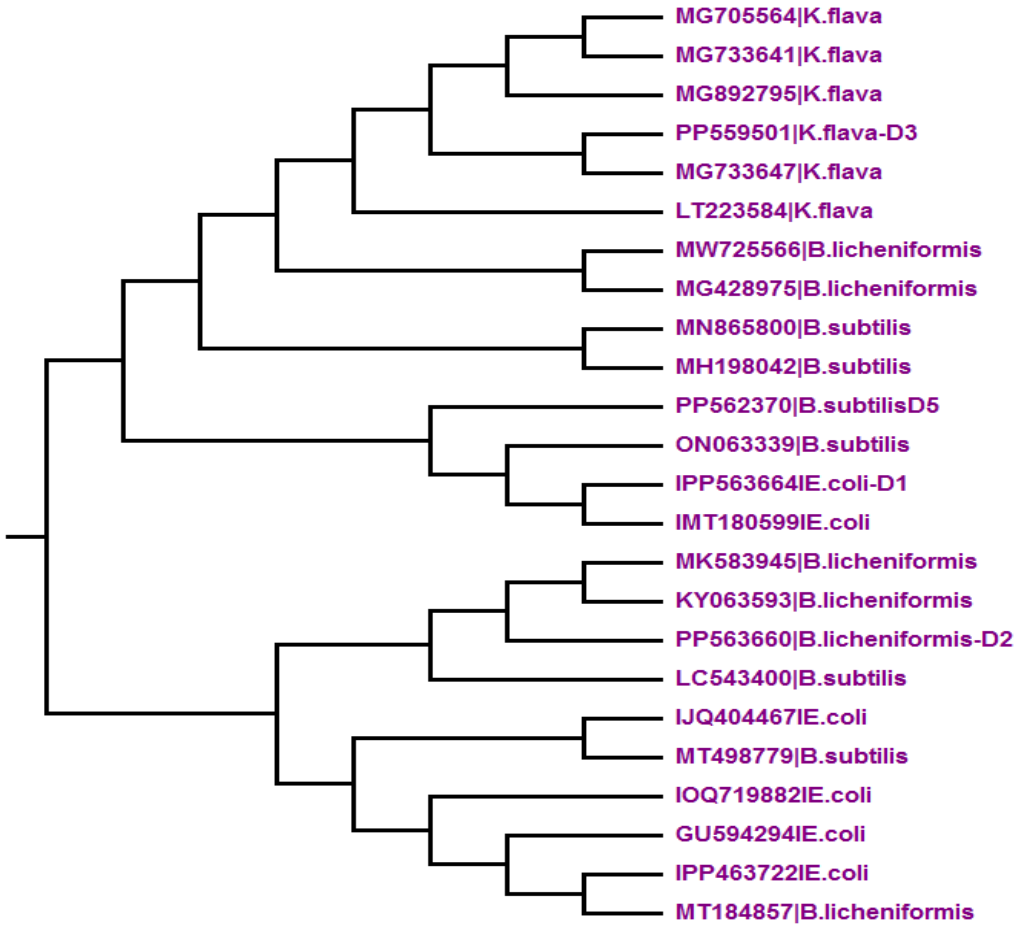

Fig 3. Phylogenetic analysis of strains isolates showing the relationships between the four isolates and 20 representative strains. Evolutionary analyses were conducted in MEGA X. Rooted phylogenetic tree (UPGMA).

Conclusion

Based on these findings, it can be concluded that a significant proportion of the soft cheese available in local markets may pose potential health risks due to microbial contamination, particularly with pathogenic bacteria such as *Salmonella* and *E.coli*. The high prevalence of these microorganisms underscores the need for targeted preventive measures and stricter quality control throughout the production and supply chain of soft cheese. To minimize these risks, it is essential for the dairy industry to adopt best practices and comply with rigorous public health standards. Key recommendations include; utilization of high-quality milk, ensuring that only milk from reliable sources, free from contamination, is used for cheese production. Implementing proper pasteurization to eliminate pathogenic and spoilage microorganisms without compromising the sensory qualities of the product is another recommendation. In addition, manufactures must follow the good manufacturing practices (GMPs) establishing strict hygiene protocols during manufacturing, packaging, storage, and transportation to prevent cross-contamination. Moreover, implementation the Hazard Analysis and Critical Control Points (HACCP) system is of key important to identify, monitor, and mitigate risks at every stage of production.

Public health authorities should consider enforcing regular inspections and microbiological testing of dairy products in local markets to ensure compliance with safety standards. Awareness campaigns targeting manufacturers, distributors, and consumers could also play a

vital role in reducing the risks associated with microbiologically unsafe cheese. By addressing these aspects, this study contributes to the broader objective of enhancing food safety standards and protecting public health, while highlighting the importance of molecular characterization techniques in identifying and managing microbial hazards in dairy products. Future work is needed to assess the pathogenicity of the isolates through advanced molecular and phenotypic analyses, ensuring a comprehensive understanding of their health implications.

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