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Prevalence of *Listeria* contamination and its relationship with microplastics and *Coliform* in dairy products: a molecular, microbiological, and statistical analysis

Sabikeh Joveinipoor,[1] Mohsen Vazifedoost,[1] Zohreh Didar,[1] Ahmad Zendehdel[2]

[1]Department of Food Science and Technology, Ne.C., Islamic Azad University, Neyshabur; [2]Department of Statistics, Ne.C., Islamic Azad University, Neyshabur, Iran

[*]**Correspondence:** Mohsen Vazifedoost, Department of Food Science and Technology, Ne.C., Islamic Azad University, Neyshabur, Iran. Tel.: +989153520388. E-mail: amirmohsen@iau.ac.ir.

Key words: *Listeria*, *actA* gene, *Coliform*, microplastics, dairy products, microbial interaction network.

Contributions: Sabikeh Joveinipoor: sampling, laboratory analyses (including PCR and total microbial tests), microplastics analysis, and microbial interaction network analysis in dairy products, statistical analyses including dendrogram construction, clustering, and statistical evaluation of microplastics data, investigation, data curation, original draft writing, editing, and supervision. Mohsen Vazifedoost: statistical analyses including frequency tables and regression analysis, editing the Results and Discussion sections, and supervision. Zohreh Didar, Ahmad Zendehdel: manuscript writing and revision. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Abstract
Listeria species are among the most significant foodborne pathogens, with dairy products providing a conducive environment for their growth and the potential development of listeriosis. This study aimed to investigate the prevalence of *Listeria* contamination in various dairy products and its relationship with *coliform* bacteria, as well as the association between *Listeria* contamination and microplastics in cheese packaging.
A total of 179 samples, including different types of cheese and butter, were analyzed using chromogenic culture media, PCR, and *actA* gene identification. The presence of *coliform* contamination in these products was also examined. Additionally, 20 brands of cheese, resembling Italian cheese types, were selected for analysis. Cotton swab sampling was performed to detect biofilms on the surfaces of cheese packaging containing microplastics.
PCR and dendrogram analysis revealed that *Listeria monocytogenes* was the most prevalent contaminant in cheese, with a strong association with the presence of the *actA* gene. Additionally, clustering and dendrogram analyses showed that *Listeria innocua* was the primary contaminant in butter samples. Microbial interaction network analysis and regression analysis further indicated a significant relationship between *Listeria* contamination and the presence of *coliform* bacteria in dairy products. Furthermore, a strong relationship was observed between *Listeria* contamination in cheese and the presence of microplastics in packaging.
The *actA* gene plays a critical role in *Listeria* infections, particularly in relation to *Listeria monocytogenes*. A significant relationship was observed between *Listeria* contamination and *coliform* presence in dairy products, underscoring the need for rigorous monitoring to prevent foodborne illness. Future studies should further explore the interactions between microbial contaminants and microplastics.

Introduction
Listeria monocytogenes (*L. monocytogenes*) is a foodborne pathogen that causes listeriosis, a group of human illnesses that occur more frequently in countries with well-developed food supply systems (Grigore-Gurgu *et al*., 2024; Manyi-Loh and Lues, 2025). The burden of foodborne illness negatively affects public health as well as national economies (Carvalho *et al*., 2023). *L. monocytogenes* is transmitted to humans and animals through the consumption of contaminated foods, including meat and meat products, fish, milk and dairy products, vegetables, and processed foods such as pizza (Adzitey and Huda, 2010; Ramaswamy *et al*., 2007). This pathogen can survive within production stages of semi-firm cheeses. Additionally, the production procedure of soft cheeses has a great potential in growing *L. monocytogenes* (Verraes *et al*., 2015).
Listeriosis, a serious illness, is commonly caused by the consumption of foods contaminated with *Listeria* species (Huang *et al*., 2015). High-risk groups include fetuses, newborns, pregnant women, the elderly, and immunocompromised individuals, accounting for approximately 30-40% of infections. People receiving organ transplants and undergoing treatment with immunosuppressive drugs are also susceptible to listeriosis (McLauchlin and Rees, 2015; Szczawiński *et al*., 2017). *L. monocytogenes* is one of the most critical foodborne pathogens associated with ready-to-eat (RTE) foods, which represent a primary vehicle for human listeriosis due to the absence of a final listericidal step and the microorganism's ability to survive and proliferate under adverse conditions (D'Ambrosio *et al.*, 2026). RTE foods have consistently been implicated in outbreaks worldwide (Niu *et al*., 2024). For example, RTE meat was a major source of listeriosis outbreaks in the United States between 1998 and 2008 (Cartwright *et al*., 2013). In July 2024, an outbreak linked to deli-sliced meat in the United States resulted in 61 confirmed cases, 60 hospitalizations, and 10 deaths across 19 states (Sharma *et al*., 2025). Additional outbreaks have been reported in Germany (2006-2008) (Winter *et al*., 2009), Switzerland (2013-2014) (Stephan *et*

al., 2015), the United States and Canada (2015-2016) (Self *et al*., 2019), South Africa (2017) (Thomas *et al*., 2020), Turkey (2022) (Şentürk *et al*., 2022), and China (2024), many of which were associated with RTE foods (Niu *et al*., 2024). Unpasteurized milk, soft cheeses, and semi-soft cheeses remain major sources of *Listeria* contamination (Mirlohi *et al*., 2015).

Recent taxonomic updates indicate that the genus *Listeria* comprises more than 20 recognized species; however, *L. monocytogenes* remains the primary pathogenic species associated with human listeriosis and foodborne outbreaks. In contrast, *Listeria innocua* is generally considered non-pathogenic, although it is frequently studied as an indicator organism due to its ecological similarity to *L. monocytogenes* and its common occurrence in food processing environments (Orsi Renato et al., 2023). It is a non-spore-forming, Gram-positive, rod-shaped bacterium that is widely distributed in the environment and readily adapts to various conditions (Murray, 2005). Characteristics such as broad pH tolerance, persistence at low temperatures, and resistance to high salinity make *L. monocytogenes* a particularly challenging pathogen in the food industry (Mahmoodi, 2010).

Conventional culture techniques for identifying bacterial infections typically require prolonged incubation periods and may not provide timely results for effective clinical decision-making. In this context, chromogenic agar (chromo agar) has emerged as a viable option. Chromo agar is a differential medium that enables the presumptive identification and confirmation of microorganisms through distinct color changes in the colonies. These color changes occur due to the interaction between specific enzymes produced by target bacteria and the chromogenic substrates incorporated into the medium. The advantages of chromogenic agar extend beyond its rapid identification capability (Rizwana *et al.*, 2025). However, the performance of culture-based methods is limited by factors such as media selection, competitive microbial growth, and subjective interpretation.

In contrast, PCR offers a more inclusive and assumption-independent approach, enabling improved detection of fastidious organisms and resolution of polymicrobial infections that conventional methods may overlook (Cummings *et al*., 2020). PCR not only confirms organisms identified by culture but can also detect additional pathogens. In many cases, pathogens detected only by PCR—previously considered false positives—may actually represent clinically significant infections missed by culture methods (Dehghani *et al*., 2026). Molecular methods such as PCR are therefore valuable for the accurate detection of pathogenic microorganisms (Xu *et al*., 2025). Compared with conventional culture techniques, PCR provides higher sensitivity and specificity by detecting bacterial DNA (Buchrieser *et al.*, 2003; Abdali *et al.*, 2018).

In recent years, PCR has gained significant attention for the detection of microbial pathogens, including *L. monocytogenes*, due to its rapidity and reliability. Accordingly, various primers have been developed to specifically identify this organism (Aygun and Pehlivanlar, 2006; Youssef *et al*., 2020; Parussolo *et al*., 2021). Numerous studies have confirmed the presence of virulence genes such as *mpl, hly, plcA, plcB, prfA,* and *actA* in *L. monocytogenes*. Among these, the *actA* gene plays an important role in controlling the interactions of actin filaments within the peripheral cytoplasm of the host cell, thereby enabling *Listeria* motility (Heinzen *et al*., 1993; Aygun and Pehlivanlar, 2006). Several studies have also reported the presence of *actA* pathogenic gene in isolates from different food types (Oliveira *et al*., 2018). In a study, researchers used PCR polymorphism to determine the length of gene fragments and the ribotyping patterns of *L. monocytogenes* in dairy products; *L. monocytogenes* strains were divided into nine distinct groups, eight of which possessed the *actA* gene, making *L. monocytogenes* (Parussolo *et al*., 2021).

L. innocua is generally considered non-pathogenic compared to *L. monocytogenes* and *L. ivanovii* due to the absence of key virulence genes (Gradovska *et al*., 2023). It has been also reported that draft whole-genome sequences were completed on five *L. innocua* isolates recovered from milk and dairy products

in Egypt. The sequencing results revealed the presence of only one antimicrobial resistance gene, *fosX*, in the *L. innocua* isolates. However, the genetic similarity between *L. innocua* and *L. monocytogenes*, along with their coexistence in similar environments, may facilitate gene transfer and potentially influence pathogenicity (Ramadan *et al*., 2023).

L. innocua is widely distributed in the environment and can survive across a broad range of pH, temperature, and salt concentration (Buchrieser *et al*., 2003; den Bakker *et al*., 2010). It is a Gram-positive, non-spore-forming, rod-shaped bacterium that can persist in environments similar to those of *L. monocytogenes*. Due to these ecological similarities, *L. innocua* may serve as an indicator of unrecognized *L. monocytogenes* contamination and potential outbreak events (Terentjeva *et al*., 2021).

Food packaging has become an important factor in food safety. Plastics such as low-density polyethylene (LDPE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and cellophane are widely used in cheese packaging, mostly conjunction vaccum due to their durability and barrier properties against moisture and oxygen (Katsara *et al*., 2025). However, these materials may release microplastics during processing and storage. Microplastics can accumulate in the human body and have been associated with inflammation, organ damage, and immune and endocrine disruption (Zajác *et al*., 2025). Moreover, microplastics provide a physical surface that readily facilitates microbial attachment and biofilm formation. Biofilm-coated microplastics can act as vectors for the persistence and transfer of microorganisms and associated contaminants within food systems—particularly in the food industry—and the environment (Zhang *et al*., 2025). The microplastic surface is prone to colonization by microbial biofilms composed of microorganisms and extracellular polymeric substances (EPS) (Chen *et al*., 2024). The slimy nature of the biofilm plays a major role in the subsequent attachment and adsorption of many other pollutants and pathogenic microbes, thereby becoming a hub or hotspot for gene transfer (Nath *et al*., 2023). The ability of *L. monocytogenes* to form biofilms on polyethylene microplastics further highlights this risk (Xu *et al*., 2024).

Recently, microbial interaction networks have been increasingly used to study relationships among microorganisms in complex ecosystems (Srinivasan *et al*., 2024).

The aim of this study was to investigate the occurrence and potential health risks of *L. monocytogenes* in dairy products using integrated culture-based and molecular methods. Specifically, chromogenic agar was used for preliminary phenotypic detection, followed by PCR-based confirmation and detection of the virulence-associated *actA* gene. In addition, the study examined the relationships between *L. monocytogenes*, *Listeria innocua*, and *coliform* bacteria within a microbial interaction framework, and assessed the potential role of microplastics as vectors influencing microbial persistence and interactions. This multifaceted approach provides a comprehensive, food safety-oriented perspective on *Listeria* detection and behavior in dairy matrices.

Materials and Methods

Materials

The materials and chromogenic culture media used in this study were supplied by Agar (France) and Merck (Germany) Companies.

Methods

Sampling

A total of 179 dairy product samples produced by various factories in Neyshabur were randomly selected. These included different types of cheese with various packaging forms, including UF cheese, processed pizza cheese, mozzarella, Bulgarian, lactic, traditional, and cream cheese, as well as pasteurized butter

and ricotta. During the experimental period, butter samples, along with processed pizza cheese and mozzarella cheese, were kept under frozen conditions, while the other samples were stored at 4°C.
Additionally, 20 cheese brands, selected based on their formulation and packaging similarities to Italian-style cheeses, were specifically examined for microplastic contamination in their packaging. Swab tests were performed at 10, 14, and 28 days of storage at 4°C.
The sample size was determined based on expected prevalence reported in previous dairy contamination studies and established food safety surveillance practices, using a 95% confidence level to ensure adequate detection of *L. monocytogenes*. All samples were aseptically collected, immediately placed in sterile containers, and transported to the laboratory under refrigerated conditions while maintaining the cold chain. Upon arrival, samples were promptly processed for microbiological analysis to minimize changes in microbial load.

Experimental design
First, conventional culture methods were used to evaluate the morphology and phenotypic identification of *Listeria* bacteria. Then, confirmatory biochemical tests were performed.
The experimental plan of the present study was designed as a stepwise, integrated approach combining classical microbiology, chromogenic culture, and molecular techniques to ensure robust and reliable detection of *L. monocytogenes* in dairy products. Initially, selective and differential culture methods were applied as a screening strategy to presumptively identify *Listeria* spp. and assess phenotypic characteristics, including invasiveness. Subsequently, chromogenic agar was employed as a standardized culture-based method to enhance specificity and enable rapid differentiation of *Listeria* species based on colony morphology and enzymatic activity. Molecular confirmation was then performed using PCR to validate the presence of *L. monocytogenes* at the genetic level, followed by targeted detection of the *actA* virulence gene to assess pathogenic potential rather than mere bacterial presence.
In parallel, coliform enumeration was conducted to provide ecological and hygienic context and to explore potential microbial associations within dairy products. Furthermore, the investigation of microplastics in cheese packaging and their biofilm-covered surfaces was integrated into the experimental design to evaluate their possible role as reservoirs or vectors facilitating microbial persistence and interaction.
This multi-layered experimental strategy was adopted to provide a comprehensive, scientifically coherent, and food safety–oriented assessment of *Listeria* contamination in dairy products.

L. monocytogenes identification
It is worth noting that all chemicals used in this study are purchased from Merck, Germany.
From each dairy product, a 25-g sample was cultured in 225 ml of *Listeria* enriched broth medium; from each sample, two cultures were prepared, one of which was kept for 48 hours at 35±2°C, while another for 15 days in the refrigerator at 4°C. Each sample was then cultured linearly on PALCAM medium as a selective medium for *Listeria*, and kept at 35°C for 48 hours; black convex colonies surrounded by a clear halo were considered as the suspected colonies. The suspected colonies were subsequently cultured on TSA plates containing 0.0015% red Congo dye and kept at 37°C for 24-48 hours. Red colonies were considered indicative of invasive strains, whereas clear or white colonies were designated as non-invasive strains. It is worth noting that this method has been used to determine the invasive phenotype of *L. monocytogenes* (Wernars *et al*., 1991; Youssef *et al*., 2020).
Subsequently, the suspected colonies were examined using Gram staining to assess bacterial morphology. Finally, oxidase testing and carbohydrate fermentation tests (rhamnose, arabinose, maltose,

mannitol, and xylose) were performed as confirmatory biochemical tests for *L. monocytogenes* (Al-Mariri *et al*., 2013; Gohar *et al*., 2017).

Bacteria culture in CHROM agar

CHROMagar culture medium was used for the identification and isolation of *Listeria* species, and the procedure was performed according to the AFNOR standard test method (Greenwood *et al*., 2005; Restaino *et al*., 1999). Initially, serial dilutions were prepared to obtain appropriate concentrations for culturing. For this purpose, samples were inoculated onto *L. monocytogenes* CHROMagar plates (CHROMagar, France) and incubated for 24 hours.

Subsequently, the cultures were incubated under microaerophilic conditions (10% CO_2) using a CO_2 incubator at 30°C. Suspected colonies were then selected after 24 hours of incubation (Rezaei *et al.*, 2019; Garcia *et al*., 2024).

Bacterial DNA extraction

Bacterial DNA was extracted using a DNA extraction kit (Fara Gene Research institute, Iran) according to the manufacturer's instructions; it was done by columnar and sedimentary methods.

Molecular characterization

The PCR method was employed for the molecular characterization of *L. monocytogenes* following a procedure similar to that described by (Elsayed *et al*., 2022). The reaction mixture (total volume 25 µL) consisted of 18.3 µL PCR-grade water, 2.5 µL 10× buffer, 1 µL of each primer, 1 µL dNTP mixture (10 mM), 1 µL DNA template (100 ng/µL), and 0.2 µL Taq DNA polymerase (1-5.2 U/µL).

Amplification was carried out in a thermocycler (Germany) with an initial denaturation step at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50.8°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

The PCR products were separated on a 1% agarose gel stained with ethidium bromide, followed by electrophoresis and visualization under UV illumination.

Detection of the actA gene

The presence of the *actA* gene was determined using PCR based on the method described by Cao *et al.* (2018). The primer sequences were first verified using the NCBI BLAST tool, then designed and synthesized by Dayan BioTech (Seoul, South Korea).

The primer sequences were as follows:

- Forward primer (*actA*-F): 5′-GCTAATAACGCAAACGGAAA-3′
- Reverse primer (*actA*-R): 5′-TAAACGCCCCTAAAGAGAAC-3′

PCR products were analyzed by agarose gel electrophoresis to confirm the presence of the target gene. The primers were synthesized at a scale of 25 nmol (OD ≈ 2).

pH measurement

The pH of samples and media was measured under standard conditions using a Metrum pH meter (Switzerland).

Coliform enumeration

Coliform counts were determined according to the AFNOR standard method (France) (Restaino *et al.*, 1999; Greenwood *et al*., 2005). The culture medium was prepared according to the manufacturer's

instructions. Samples were cultured using the pour plate method and incubated at 37°C for 24 h (Rezaei *et al.*, 2019).
After incubation, red colonies were identified as *coliforms*, while blue colonies were identified as *E. coli* (Restaino *et al*., 1999; Greenwood *et al*., 2005; Vlaemynck *et al*., 2000; Scotter *et al.*, 2001; Garcia *et al*., 2024).

Detection of microplastics
The presence of microplastics in plastic-based food packaging and dairy products has already been well established in recent high-quality studies using validated analytical techniques such as FTIR, Raman microscopy, and spectroscopic imaging (Giri *et al*., 2024; Visentin *et al*., 2024; Santonicola *et al.*, 2025; Xu *et al.*, 2025; Zajác *et al*., 2025). To avoid unnecessary repetition of previously validated analytical methods and to preserve the novelty of the present work, we did not repeat microplastic identification protocols.
Instead, packaging materials that are well documented in the recent literature to contain microplastics were selected, and the focus of the study was placed on a novel and underexplored aspect: the microbiological interaction between *L. monocytogenes*, *Listeria innocua*, and microplastic-containing packaging surfaces, including bacteria migration, surface colonization, and biofilm formation during refrigerated storage.
Surface sampling of the inner packaging layer was conducted to evaluate the presence and behavior of *L. monocytogenes* and *L. innocua* on these materials. A cotton swab test was performed to detect *Listeria* contamination on microplastic-associated surfaces in plastic cheese packaging, where biofilm formation was observed as slimy layers or sediment (Jansson *et al*., 2020; Chen *et al*., 2024).
This approach allowed us to specifically address the food safety and public health implications of microplastics as potential vectors for pathogenic bacteria, rather than focusing on their analytical detection, which has already been comprehensively addressed in recent literature.

Statistical method
Similarity and pattern matrices, represented by two-dimensional dendrogram diagrams, were used to investigate *Listeria* contamination patterns in dairy products from different factories in Neyshabur. A hierarchical clustering approach, integrated with principal component analysis (PCA) and supported by statistical tests, was applied to provide a reliable and biologically meaningful framework for characterizing and differentiating dairy products based on *Listeria* contamination patterns and related parameters.
Principal component analysis (PCA) is a multivariate statistical method that simplifies complex datasets by transforming correlated variables into a smaller number of uncorrelated components, thereby highlighting patterns and groupings in the data (Jalaei Salmani *et al*., 2023; Mansouri *et al*., 2022; Jolliffe, 2025). Similarity analysis and dendrogram construction were performed using standard hierarchical clustering methods, based on the presence or absence of the target gene and physicochemical variables such as pH. This approach is widely accepted in molecular epidemiology studies of bacterial isolates.
In addition, regression analysis and microbial interaction network analysis (Spearman and Kendall correlations) were used to evaluate the relationship between *coliform* presence and *Listeria* survival. A K^2 test was also performed to assess the association between *Listeria* contamination and the presence of microplastics.

Results

The experimental results of the studied dairy products are presented in Table 1, which includes samples obtained from nine factories in Neyshabur, comprising 21 codes for different types of cheese and five codes for butter (*Supplementary Tables 1 and 2*).

Investigation of Listeria contamination frequency in various dairy products of Neyshabur

The results showed that the highest contamination with *L. monocytogenes* was observed in ricotta and pasteurized cheese, while the lowest contamination was found in lactic cheese. Additionally, pasteurized cheese exhibited the highest contamination with *Listeria innocua* (Figure 1).

Prevalence of Listeria in dairy products and microplastic packaging

Swab tests from cheese packaging surfaces over storage periods of 10, 14, and 28 days at 4 °C demonstrated notable migration of *Listeria* from cheese to packaging materials containing microplastics. The main findings were as follows:

- Ricotta packaging: 100% contamination with both *L. monocytogenes* and *L. innocua*
- Mozzarella packaging: 90% (*L. monocytogenes*), 70% (*L. innocua*)
- Pasteurized cheese packaging: 90% (*L. monocytogenes*), 60% (*L. innocua*)
- Processed pizza cheese packaging: 90% (*L. monocytogenes*), 70% (*L. innocua*)
- Bulgarian cheese packaging: 100% for both species
- Cream cheese packaging: 70% (*L. monocytogenes*), 10% (*L. innocua*)

These findings indicate a significant risk of microbial migration from cheese surfaces to microplastic-containing packaging materials, particularly during cold storage.

Frequency of Listeria contamination and transfer to microplastics

The results presented in Table 1 and *Supplementary Figure 1* show that *Listeria* contamination was transferred from contaminated cheeses to microplastic-containing packaging surfaces over 10, 14, and 28 days at 4 °C.

Swab test results demonstrated an increasing trend in *Listeria* contamination on microplastic surfaces, corresponding to the level of contamination in the cheese samples.

Correlation between coliforms and L. monocytogenes

Regression analysis indicated a strong positive correlation between *coliform* counts and *L. monocytogenes* contamination (R^2=0.89). As *coliform* levels increased, *L. monocytogenes* contamination also increased (Figure 2A).

Correlation between coliforms and L. innocua

No significant linear correlation was observed between *coliforms* and *L. innocua* (R^2 = 0.13), indicating a weak relationship between these variables (Figure2B).

Importance of Listeria contamination in cheese and ricotta

Dendrogram analysis showed that *L. monocytogenes* contamination was significantly higher in cheese products. Higher pH values were associated with increased *L. monocytogenes* contamination (Figure 3A).

Importance of Listeria contamination in various types of butter

The analysis indicated that *L. innocua* contaminaton in butter samples was higher than *L. monocytogenes*, particularly in pasteurized butter (Figure3B).

Discussion

Investigating the frequency of Listeria contamination in various dairy products of Neyshabur

In this study, various types of dairy products were investigated for *L. monocytogenes* contamination. Among the 179 analyzed samples, 64.2%, 50%, 28.57%, 22.22%, 38.46%, 53.84%, 60%, 58.33%, and 68.75% of mozzarella, processed pizza, cream, UF, lactic, pasteurized, Bulgarian, traditional, and ricotta cheeses, respectively, were contaminated with *L. monocytogenes* (Table 1).

It should be noted that food processing prior to consumption may lead to bacterial death; however, the presence of dead cells can result in false-positive PCR results. To address this limitation, several studies have applied enrichment culture prior to PCR to increase the number of viable bacteria and improve detection sensitivity (Azinheiro *et al*., 2022). Moreover, employing enrichment culture medium leads to the dilution of dead bacteria that are the inhibiting agents of PCR reaction (Ricchi *et al*., 2017).

The results of the present study showed that approximately 70% of the analyzed dairy samples contained the *actA* gene, indicating the pathogenic potential of *L. monocytogenes*. This gene plays a critical role in actin polymerization, enabling intracellular and intercellular movement, escape from the phagolysosome, resistance to host immune defenses, and replication within host cells. The *actA* gene is therefore essential for *Listeria* motility and virulence (Parussolo et al., 2021). Previous studies have also reported that the resistance of the actA gene in *L. monocytogenes* increases the prevalence of Listeria contamination in industrial foods (El-Zamkan *et al.*, 2021). Understanding the genetic characteristics of *L. monocytogenes* is therefore essential for controlling this pathogen and reducing the risk of listeriosis (Wiktorczyk-Kapischke *et al*., 2023).

L. monocytogenes is responsible for listeriosis, a disease with a high mortality rate (up to 30%). This pathogen is highly tolerant to environmental conditions, with a growth range between 0°C and 42°C and an optimal temperature of 30-35°C. Although growth slows below 5 °C, the bacterium can still survive under refrigeration conditions. In addition, it tolerates a wide pH range and low nutrient availability and is widely distributed in the environment, including water, soil, dairy, meat, and vegetables (Wiktorczyk-Kapischke *et al.*, 2023).

In the present study, *Listeria* contamination was not observed in cheeses produced using industrial ultrafiltration methods. Contamination of dairy products may occur due to ineffective pasteurization or post-processing contamination, particularly during packaging, handling, or storage (Wei *et al*., 2024). These findings are consistent with the results of the present study (Figure 4).

Investigating the correlation between the presence of coliforms and L. monocytogenes contamination in dairy products

L. monocytogenes and *coliform* bacteria are both capable of growing in milk and dairy products (Dal Bello *et al.*, 2012). They can also survive under refrigerated conditions and in humid environments (Lewis *et al*., 2006). The presence of certain microorganisms in cheese may indirectly promote the growth of *L. monocytogenes*, for example through pH changes that create more favorable conditions (Schvartzman *et al*., 2014), as shown in *Supplementary Figure 2*.

Due to similarities between *coliforms* and *Listeria* spp. in terms of environmental adaptability and growth characteristics, their relationship was evaluated using regression analysis, as demonstrated in Figure 2A.

Coliforms are facultative aerobic and anaerobic bacteria, capable of growing over a wide temperature range (10-46°C), utilizing diverse substrates, and producing acid and gas from sugars. Similarly, *L.*

monocytogenes is a facultative anaerobe with high environmental adaptability, able to survive across a broad range of temperatures and conditions (Buchrieser *et al*., 2003).
These shared physiological traits support the hypothesis of co-occurrence and possible interaction within dairy matrices. The results of this study are consistent with previous findings and demonstrate a significant positive correlation between *L. monocytogenes* and *coliform* counts. According to Table 2 both Spearman and Kendall's tau tests confirmed a statistically significant relationship ($p<0.05$), indicating that an increase in *coliform* levels is associated with increased *L. monocytogenes* contamination.

Investigating the transmission and relationship of Listeria contamination with microplastics
Techniques used to identify microplastics in food packaging include thermal methods, Raman spectroscopy, scanning electron microscopy (SEM), Fourier-transform infrared (FTIR) spectroscopy, attenuated total reflectance FTIR (ATR-FTIR), focal plane array (FPA)-FTIR, near-infrared spectroscopy (NIR), and nuclear magnetic resonance (NMR) spectroscopy (Giri *et al*., 2024; Santonicola *et al*., 2025).
In recent years, these techniques have been widely applied to detect microplastics in the packaging of various milk and cheese products (Kiruba *et al.*, 2022; Zipak *et al*., 2022; Buyukunal *et al*., 2023; Kaseke *et al.*, 2023; Giri *et al*., 2024; Chinglenthoiba *et al*., 2025; Katsara *et al*., 2025; Santonicola *et al*., 2025). Therefore, repeating these analytical methods to confirm the presence of microplastics in cheese packaging was not considered in this study.
Microplastics can selectively accumulate microorganisms from their surrounding environment, promoting biofilm formation. The researchers provided important insights into how pathogens form biofilms on microplastics, highlighting their role in microplastic-biofilm contamination and the potential transfer of pathogenic bacteria into the human body (Xu *et al*., 2024). Control efforts are further complicated by the ability of *L. monocytogenes* to form biofilms and persist under cold storage conditions (Radoshevich and Cossart, 2018; Sharma *et al*., 2025).
In this study, a cotton swab method was used to detect *Listeria* contamination on microplastic surfaces in plastic cheese packaging (Chen *et al*., 2024). This method is suitable for evaluating bacterial contamination on surface materials. Microplastic contamination in food has gained increasing attention due to its potential health impacts, including oxidative stress, inflammation, carcinogenesis, neurodegeneration, immune system disruption, and altered energy metabolism (Giri *et al*., 2024).
Here, swab sampling was performed on biofilm-covered inner packaging surfaces of cheese samples. These surfaces covered with slimy layers of sediment or biofilm, that allow *L. monocytogenes* and *L. innocua* adhered , mutated and exchanged (Jansson *et al*., 2020). Fat-rich cheeses such as cream cheese, mozzarella, and processed pizza cheese, which contain high fat and 1-1.5% salt, are comparable to Cretan Graviera cheese. A recent work has shown that microplastics can migrate from LDPE and PP packaging to cheese surfaces, with microbial growth more pronounced in LDPE than in PP packaging. This study suggests that microplastics act as substrates for microbial attachment and biofilm formation, facilitating the persistence and potential transfer of foodborne pathogens. These particles can function as vectors by adsorbing bacteria and enabling their survival under adverse environmental conditions, thereby contributing to cross-contamination along the food processing and packaging chain. Although the role of microplastics in biofilm-mediated transmission of *Listeria* spp. remains insufficiently explored, this emerging mechanism highlights a potential link between packaging-derived microplastics and microbial food safety risks (Katsara *et al*., 2025).

According to *Supplementary Table 3*, there is a significant relationship between these two types of *Listeria* contamination in microplastic biofilms. Moreover, *Supplementary Figure 1* shows that *L. monocytogenes* contamination is higher in microplastic biofilms.

Microplastics as vectors for Listeria transmission

One of the key findings of this study is the demonstrated transfer of *Listeria* from contaminated cheese to the inner surfaces of plastic packaging materials. Packaging composed of LDPE, PP, and cellophane showed clear evidence of biofilm formation and microbial adhesion during cold storage.

Swab test results revealed that contamination—particularly by *L. monocytogenes*—increased over time, indicating progressive migration and colonization of packaging surfaces. These findings suggest that microplastics are not merely passive contaminants but may actively support microbial persistence and dissemination.

While previous studies using Raman and FTIR spectroscopy have confirmed the migration of microplastics into food products, the present study provides complementary microbiological evidence of their role in facilitating bacterial transfer. This highlights the importance of considering microplastics as potential vectors in food safety risk assessments.

Investigating the correlation between the presence of coliforms and L. innocua contamination in dairy products

L. innocua is a saprophytic, hemolytic bacterium widely distributed in the natural environment, commonly found on the surfaces of water, vegetables, and food waste. It can survive across a broad range of pH, temperature, and salt concentration (Moreno-Enriquez *et al*., 2007). In the plasmid of *L. innocua*, there is a compound called bacteriocin, which is a type of antimicrobial peptide. Bacteriocin is a small peptide molecule composed of 30 to 60 amino acids. It is generally produced by lactic acid bacteria and other species. Bacteriocin is beneficial for controlling *L. monocytogenes*; therefore, it can reduce the pathogenic risk associated with food (Banerjee *et al*., 2004).

However, various genetic mechanisms of virulence transfer among *Listeria* species and other genera could raise the possibility of the evolution of virulent strains of *Listeria innocua*. Specifically, the presence of CLPL carried on plasmids alongside common virulence and antimicrobial resistance genes—combined with ongoing genetic mechanisms of virulence transfer among *Listeria* and other genera—could endanger current industrial processing and preservation protocols and present health risks associated with dairy products (Ramadan *et al*., 2023). Such mechanisms may compromise current food processing and preservation strategies and pose public health risks.

In this study, *L. innocua* was detected in 75%, 81.25%, 28.57%, 25.92%, 23.69%, 100%, 60%, 50%, and 100% of mozzarella, processed pizza, cream, UF, lactic, pasteurized, Bulgarian, traditional, and ricotta cheeses, respectively, as demonstrated in Figure 3.

Statistical analysis showed no significant linear relationship between *L. innocua* and *coliform* counts based on regression analysis. However, non-parametric tests (Spearman's and Kendall's tau) revealed a significant positive association ($p<0.05$). This discrepancy suggests that the relationship between *L. innocua* and coliforms is likely non-linear or more complex than can be captured by linear regression models.

Investigating the importance of Listeria contamination in various types of cheeses and ricotta based on dendrogram and clustering analysis

Dendrogram and clustering analyses revealed that *L. monocytogenes* contamination had the highest contribution among cheese products. The results also indicated that pH is a key factor influencing

bacterial growth, with higher pH values promoting increased *L. monocytogenes* proliferation (Schvartzman et al., 2014).
These findings are consistent with previous studies (Cole *et al*., 1990; Scotter *et al*., 2001) demonstrating the significant role of pH in supporting *Listeria* growth in dairy matrices, as demonstrated in *Supplementary Figure 2*.

Investigating the importance of Listeria contamination in various types of butter based on dendrogram and clustering analyses

Clustering analysis identified two main groups among butter samples. Cluster 1 included pasteurized butter samples with a similarity index of 0.68, while Cluster 2 also included pasteurized butter with a similarity index of 0.65. Notably, butter samples from factories 1 and 6 showed high similarity within Cluster 1 (similarity index = 0.89), compared to samples from factory 3.
The clustering pattern indicates that *L. innocua* was the dominant factor influencing group formation in butter samples. In Cluster 2, *L. monocytogenes* growth was limited, whereas *L. innocua* contamination was relatively higher. This suggests that *L. innocua* may play a more significant role in shaping microbial profiles in butter products.
Given the ability of *L. monocytogenes* to survive and persist under low-temperature conditions, its presence in butter remains noteworthy (Bouymajane *et al*., 2021).
Statistical analysis (Spearman and Kendall's tau) indicated a borderline but significant relationship between *L. monocytogenes* and *coliforms* ($p<0.05$), with positive coefficients suggesting that increases in *coliform* counts are associated with increased *L. monocytogenes* contamination. A similar significant positive relationship was observed between *L. innocua* and *coliforms*.

Investigating the importance of Listeria contamination in microbial interaction networks in dairy

Microbial interaction networks represent a fundamental framework for understanding complex microbial ecosystems. By characterizing interactions as positive, negative, or neutral, these approaches provide insight into how microbial species influence each other within a given environment.
Such interactions can be studied using both qualitative methods—such as co-culture experiments combined with microscopy and multi-omics approaches (metagenomics, metabolomics, and metatranscriptomics)—and quantitative methods, including network construction and computational modeling. Statistical tools such as Spearman and Kendall's correlations are commonly used to infer relationships from microbial abundance data (Srinivasan *et al*., 2024).
In the present study, microbial interaction network analysis demonstrated a significant association between *Listeria* contamination and *coliform* presence in dairy products. This finding highlights the importance of considering microbial communities as interconnected systems rather than isolated organisms, and supports a more integrated approach to food safety assessment.

Conclusions

This study identified and characterized *Listeria* contamination in various cheeses and butters produced in Neyshabur, Iran. Bacteriological analysis and PCR assays revealed significant contamination by *L. monocytogenes* in several dairy products, including cheeses, ricotta, and industrial butter, with a substantial proportion carrying the invasive *actA* gene.
Cluster (dendrogram) analysis indicated that *L. monocytogenes* was the dominant contaminant in cheeses, whereas *L. innocua* was more prevalent in butter samples. Principal component analysis (PCA) further confirmed *L. monocytogenes* as the most influential contaminant and suggested a positive association with higher pH values.

Regression analysis showed a correlation between *L. monocytogenes* and *coliform* counts, while no linear relationship was observed between *coliforms* and *L. innocua*. However, non-parametric tests (Spearman's rho and Kendall's tau) indicated a significant positive, though non-linear, association between these variables. This discrepancy suggests that the relationship between microbial populations may be complex and not adequately captured by linear models alone. In this study, the role of microplastics as vectors that facilitate microbial persistence and interaction was evaluated and confirmed. The findings related to microplastics highlight the need for improved hygienic practices, stricter control of packaging materials, and enhanced monitoring of dairy production environments. Future research should employ advanced molecular and spectroscopic techniques to better elucidate the interactions between microbial contaminants and food contact–related factors, including microplastics, in dairy systems.

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Online supplementary material

Table 1. Distribution of *Listeria* contamination in various dairy products.

Type of dairy product	Total number of samples	The number of samples contaminated by *L. innocoa*	The number of samples contaminated by *L. monocytogenes*	The number of samples contaminated by *Listeria*
Mozzarella cheese	28	24 (85.70%)	18 (64.20%)	21 (75%)
Processed cheese	16	12 (75%)	8 (50%)	13 (81.25%)
Cream cheese	21	9 (42.85%)	6 (28.57%)	6 (28.57%)
UF cheese	27	9 (33.33%)	6 (22.22%)	7 (25.92%)
Lactic cheese	13	9 (69.23%)	5 (38.46%)	9 (69.23%)
Pasteurized Butter	28	23 (82.14%)	18 (64.28%)	20 (71.42%)
Pasteurized cheese	13	13 (100%)	7 (53.84%)	13 (100%)
Bulgarian cheese	5	3 (60%)	3 (60%)	3 (60%)
Traditional cheese	12	10 (83.33%)	7 (58.33%)	6 (50%)
Ricotta	16	16 (100%)	11 (68.75%)	16 (100%)

Table 2. Relationship between *L. monocytogenes* and *L. innocua* with *coliform* counts in cheese and butter samples based on Spearman's rho and Kendall's tau-b tests.

Pathogens	Product Type	Spearman's	Kendall's taub	P
Relationship between *L.monocytogenes* and Coliform	Cheese	0.380	0.319	<0.05
Relationship between *L.innocua* and Coliform	Cheese	0.387	0.316	<0.05
Relationship between *L.monocytogenes* and Coliform	Butter	0.339	0.278	0.05
Relationship between *L.innocua* and Coliform	Butter	0.442	0.372	<0.05

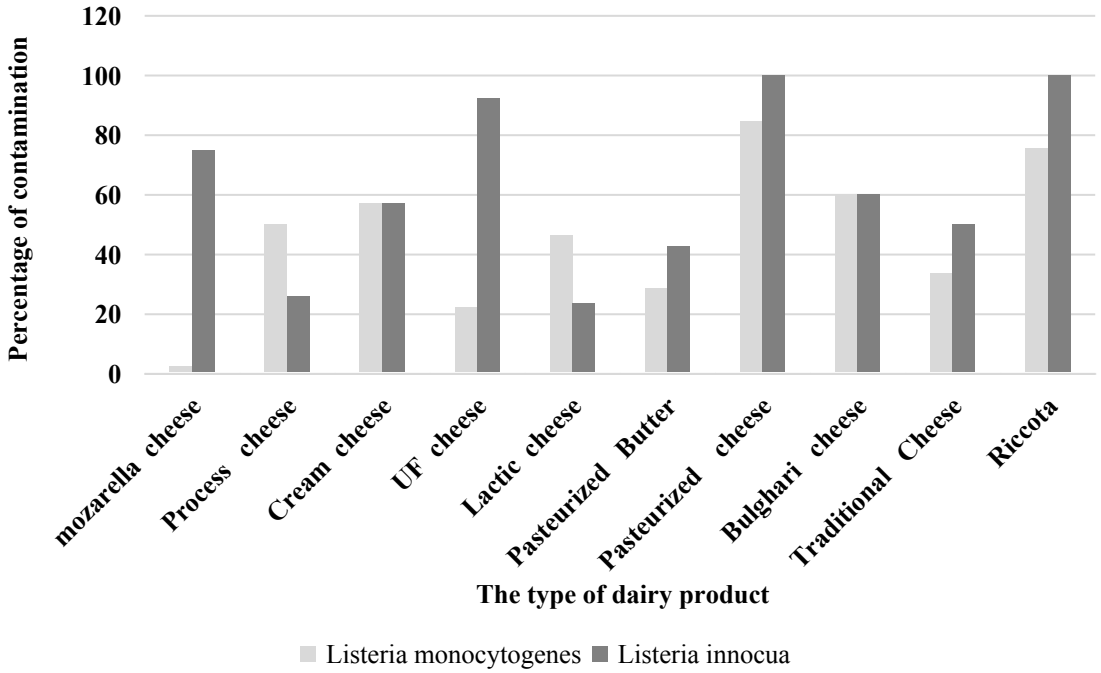
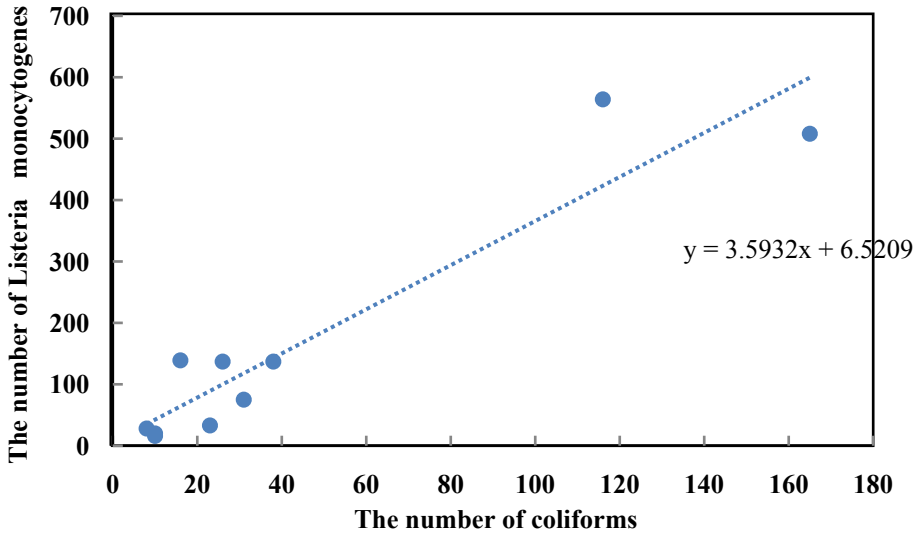
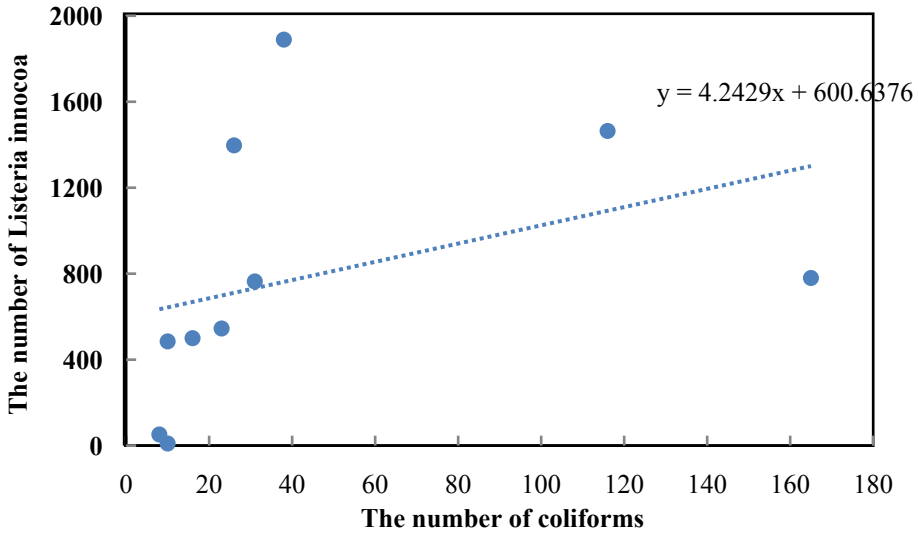


Figure 1. The amount of contaminated dairy products by *Listeria*.

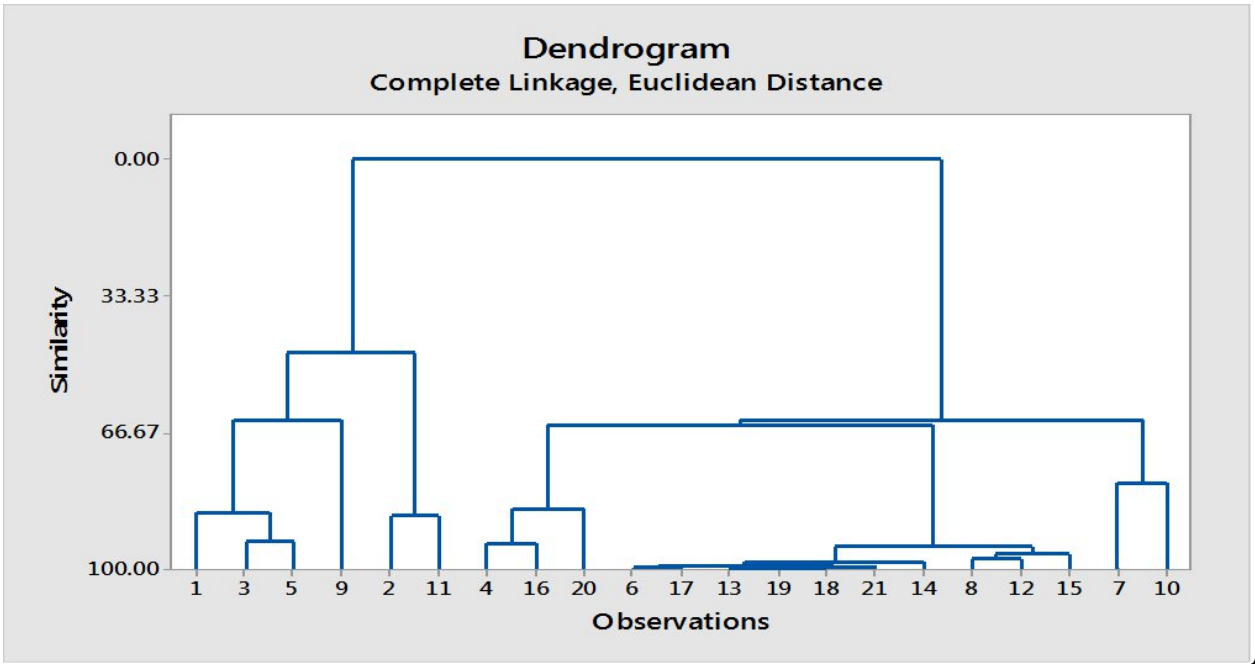


A

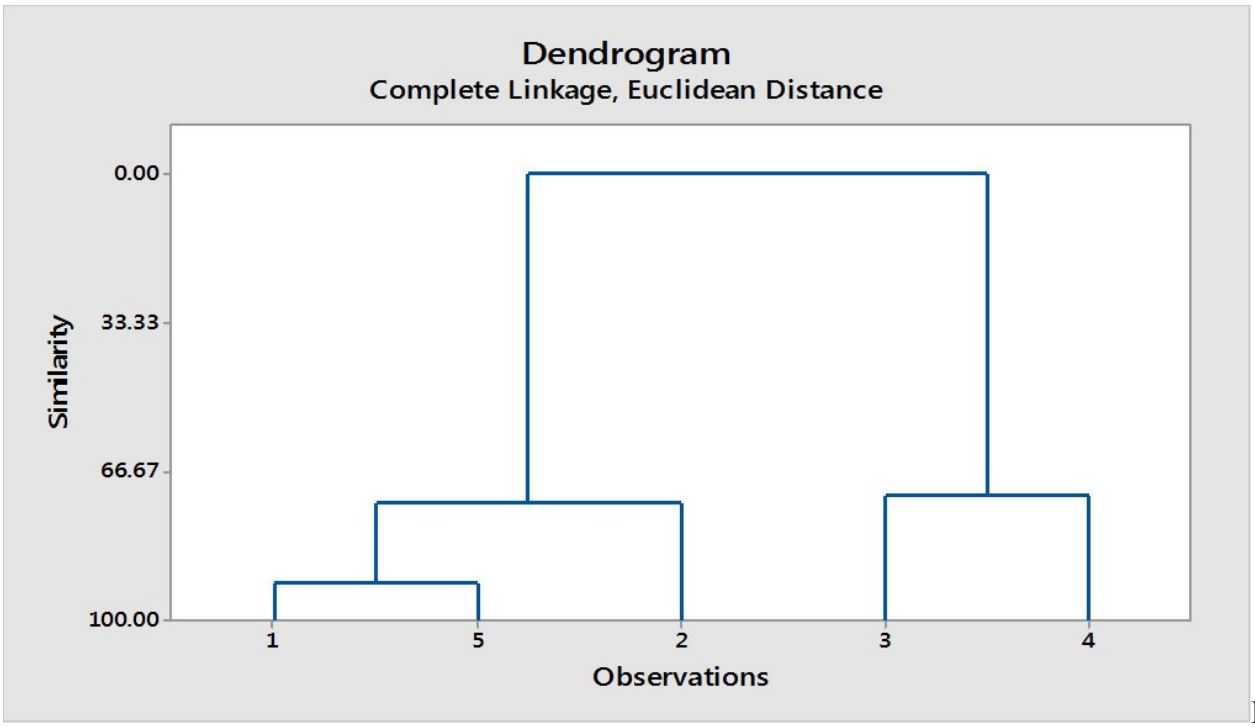


B

Figure 2. A). Correlation between *coliform* counts and *L. monocytogenes* contamination in dairy products; B) correlation between *coliform* counts and *L. innocua* contamination in dairy products.

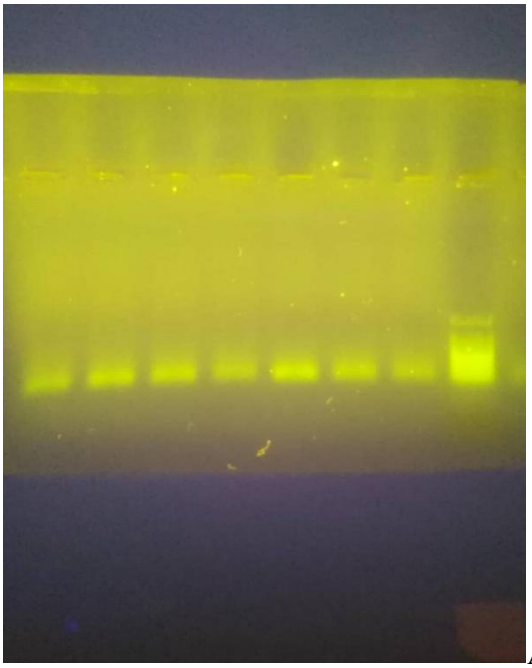


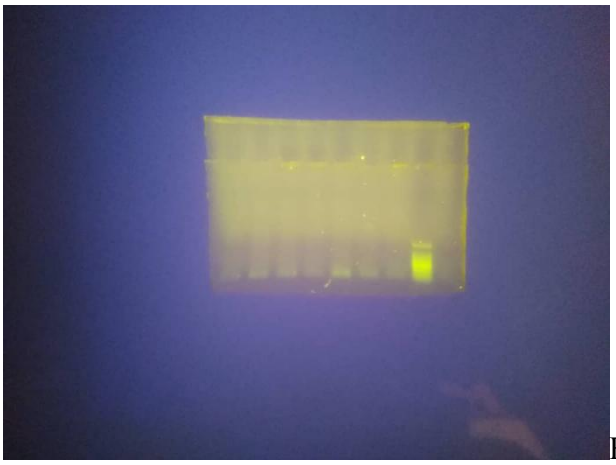
A



B

Figure 3. A). Diagram of dendrogram clustering for various types of cheeses and ricotta (cluster 1: saffron mozzarella cheese of factory 1, ricotta of factory 1, mozzarella cheese of factory 1, pasteurized cheese of factory 4 with similarity index = 0.63; cluster 2: ricotta of factory 2, saffron process cheese of factory 1 with similarity index = 0.89; cluster 3: traditional cheese of Market, UF cheese of factory 1, Lactic cheese of factory 1 with similarity index = 0.80; cluster 4: Bulgarian cheese of factory 2, cream cheese of factory 5, cream cheese of factory 6, UF cheese of factory 2, UF cheese of factory 6, UF cheese of factory 8, lactic cheese of factory 2 with similarity index = 0.90; cluster 5: saffron mozzarella cheese of factory 3, process cheese of factory 1 with similarity index = 0.76); B) diagram of dendrogram clustering for butter samples


A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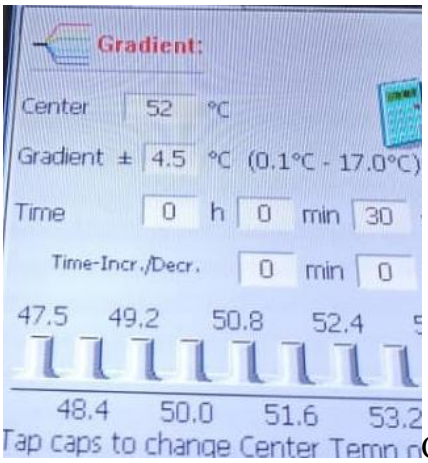

C

Figure 4. A) PCR for *L. monocytogenes*; B) representation of best band of *actA* gene; c) protocol temperature range in PCR.