

The prevalence of some pathogenic bacteria in traditional cheese sold in local markets in Sulaimani, Iraq

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Abstract

Cheese is an important dairy product that is widely consumed worldwide. However, it can become contaminated with foodborne pathogens during production, processing, handling, or storage, posing a potential risk to public health. The aim of the present current study was to investigate the occurrence of major foodborne pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella spp.*, in some cheese samples sold in Sulaimani city of Iraq. To achieve this, sixty-three cheese samples were collected from retail markets and analyzed using conventional culture methods. The results confirmed using biochemical tests (VITEK 2), and Molecular based method using polymerase chain reaction (PCR) targeting species specific primers targeting *hlyA*, *16S rRNA* genes in *Listeria monocytogenes*, *Nuclease genes*, *16S rRNA* genes in *staphylococcus aureus* and *invA*, *hlyA* (SPI-1) gene in *salmonella spp.*. The results showed that *Staphylococcus aureus* had the highest prevalence (42.85%) among the tested pathogens in cheese samples, followed by *Listeria monocytogenes* (4.8%), while *Salmonella spp.* showed the lowest prevalence (3.17%). Identification of *S. aureus* using VITEK 2 AST-GP79 card was successfully done with a good to acceptable level of confidence (90 – 99%), and for *L. monocytogenes* the rate of probability of identification was 97%, and for *Salmonella* was 98%. Identification of the studied bacteria and assessment of their virulence potential was successfully confirmed by PCR analysis. *S. aureus* isolates were identified by the amplification of *16S rRNA gene* (750 bp) and *nuc* gene (276bp). *L. monocytogenes* were identified by the amplification of *16S rRNA gene* (938bp) and *hlyA* genes (701bp). PCR assay also confirmed the presence of *Salmonella spp.* by amplification of the *invA* (275bp) and *hlyA* gene (768bp). All these techniques provided a reliable and complementary approach for the identification of bacterial pathogens in the studied cheese samples. The present results indicated that traditional cheeses manufactured in Sulaimani were not safe microbiologically. Regular bacterial detection of food born bacteria in cheese is needed by combining culturing method with more accurate rapid molecular detection technique.

Keywords: Cheese, contamination, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella spp.*

Introduction

Cheese is a fermented dairy product with high nutritional value that make it one of the most important ready-to-eat foods produced worldwide. The global market value of cheese by 2027 is expected to

exceed \$113,000 million. There are numerous varieties of cheese that differ in their texture and flavor depending on factors such as the type and composition of milk, the animal species, animal feeding practices, and the methods used for cheese production. Traditional cheeses are often produced using artisanal methods, which

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may involve limited standardization of hygienic and processing practices. Consequently, some traditionally produced cheeses have been associated with foodborne illness outbreaks. While these cheeses are valued for their unique flavors and textures, they can also serve as potential vehicles for bacterial contamination, due to factors such as inadequate pasteurization, poor sanitation, improper storage conditions with minimal standardized hygiene controls and sometimes using raw milk which can facilitate contaminate pathogenic microorganisms [39, 46]. Among these pathogens, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella spp.* Are particularly significant because they are often linked with dairy foods, they can lead to very serious illness in humans and they are known to regulatory bodies to be microbiological hazards. *Staphylococcus aureus* is one of the bacterial species able to contaminate cheese from infected dairy animals or through poor hygiene in cheese handling and can produce heat-stable enterotoxins which can lead to food poisoning [30]. Because *Listeria monocytogenes* is a psychotropic bacterium which can grow and survive at refrigeration temperatures hence it can persistent and remain a potential source of contamination in cheese products. It is the causative agent of listeriosis, a severe disease that is particularly dangerous for neonates, pregnant women, elderly and immunocompromised individuals. [32]. *Salmonella spp.*, which are known for their zoonotic potential, may also contaminate cheese by using raw milk or fecal contamination or by inadequate hygienic practices, and cause many outbreaks of salmonellosis [42]. Thus, proper detection and identification of these pathogens in cheese is vital for its risk assessment and consumer safety.

Numerous studies have been performed to detect and identify these pathogens in dairy

products with different techniques such as conventional (culture-based) methods which are considered as the gold standard in microbial detection; however they are time-consuming and may not be sensitive enough in complex food matrices. Hence, immunological assays and biosensors have been found to be useful for on-site testing and detection of pathogenic bacteria in cheese, in different geographical areas [2,16,33].

Furthermore, polymerase chain reaction (PCR) using species specific genes are molecular techniques that provide the ability to identify these bacteria in a rapid, sensitive and specific manner, important for prompt intervention [61]. Moreover, the reported prevalence of enterotoxin genes in cheese isolates highlight the need for molecular tools for species identification as well as virulence assessment.

In Sulaimani city of Kurdistan Region of Iraq, the production of some traditional cheeses may rely on the natural microflora of raw milk rather than using pasteurization and standardized commercial starter cultures. [40], which poses great food safety concerns [27]. Hence Standardizing detection procedures and enhancing hygienic practices for domestic cheese, are crucial for minimising risks for foodborne illnesses safeguarding public health. Due to the limited comprehensive data available regarding the current safety status of these types of cheese, this study was conducted to assess the microbiological safety of various traditional cheese samples collected from the Sulaimani market focuses on the detection and identification of *S. aureus*, *L. monocytogenes*, and *Salmonella spp.*, by combining traditional, biochemical and PCR-based approaches, in order to assess microbiological safety and potential public health risks.

2. Materials and Methods

2.1. Sample collection:

A total of 63 traditional cheese samples, representing different cheese types were collected from retail markets and local shops in Sulaimani Province, Kurdistan Region, Iraq, between December 2024 and June 2025. Samples were transported to the laboratory under refrigerated conditions and stored at 4 °C until microbiological analysis. Each cheese sample divided into four parts, each with 25-g, that aseptically weighed into individual sterile Whirl-Pak® bags. Three aliquots were used for the isolation and detection of *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp., whereas the remaining portion was reserved for physicochemical quality analyses.

2.2. Isolation of selected bacteria in cheese samples used in the current study:

2.2.1. Isolation of *Staphylococcus aureus*

Isolation and enumeration of *Staphylococcus aureus* were performed according to [26]. Ten grams of each cheese sample was homogenized in 90 mL of sterile Peptone Water (Oxoid CM0009). Three aliquots (333 µL) of the homogenate were spread onto individual Baird–Parker agar plates supplemented with Egg Yolk Tellurite Emulsion (EYT) (Oxoid, UK) and incubated at 35 °C for 48 h. When necessary, decimal serial dilutions were prepared to obtain countable plates. Typical colonies were subsequently streaked onto Baird–Parker agar supplemented with Rabbit Plasma Fibrinogen (RPF) (Oxoid, UK) and incubated at 35 °C for 48 h. Colonies exhibiting characteristic black or gray pigmentation surrounded by an opaque precipitation halo were considered presumptive coagulase-positive *S. aureus* [43].

2.2.2. Isolation of *Listeria monocytogenes*

For the presence of *L. monocytogenes* a modification of the standard method described in the US Food and Drug Administration Bacteriological Analytical Manual (US FDA-BAM) by [23] was used. Twenty-five g of each cheese sample was taken aseptically and homogenized in 225 mL of buffered listeria enrichment broth (BD Difco, USA) for 1 min using a stomacher (Bagmixer-interscience, France) and incubated at 30°C. Then *Listeria* selective enrichment supplement (Oxoid, UK) was added after 4-h incubation, Enrichment was continued for 48 h overall. Then 50 µL of the enrichment culture was streaked onto *L. monocytogenes* Plating Medium (LMPM) and incubated at 35°C, for 48 h. Presumptive colonies exhibiting typical morphology were repeatedly sub-cultured on fresh media until a pure culture was obtained for further identification.

2.2.3. Isolation of *Salmonella* spp.

Isolation of *Salmonella* spp. was carried out according to the FDA-BAM method with minor modifications described by [57]. A pre enrichment was prepared by homogenizing a 25-g of each cheese sample in 225 mL of lactose broth and incubated at room temperature for 1 h, followed by incubation at 37 °C for 24 h. Subsequently, 1 mL of the pre-enrichment culture was transferred into 9 mL of each of two selective enrichment medium (Tetrathionate Broth (Oxoid, UK) Rappaport–Vassiliadis medium (BD Difco, USA). Both selective enrichment broths were incubated at 42 °C for 24 h in a shaking water bath. Then 50 µL aliquots from each broth were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Neogen, USA) and CHROMagar™ *Salmonella* (CHROMagar, France), followed by incubation at 37 °C for 24 ± 3 h. Typical colonies were purified by repeated streaking before further characterization.

2.3. Species Identification Using the VITEK® 2 System

Phenotypically identified *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella spp.* isolates were used for species-level identification using the automated VITEK® 2 system (bioMérieux, Marcy l'Étoile, France).

Fresh bacterial culture (18–24 h) grown from single colony of each bacterium was suspended in sterile saline solution (0.45–0.50% NaCl) and the turbidity was adjusted to equal 0.5 McFarland standard, using spectrophotometer. Subsequently, 145 µL of the standardized bacterial suspension was transferred into a tube containing 3.0 mL of sterile 0.45% saline solution. The prepared suspension tubes were then loaded into the VITEK® 2 system according to the manufacturer's instructions [11].

ID-GP cards were used for identification of Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and AST-GN97 identification cards were used for Gram-negative bacteria (*Salmonella spp.*). The cards include several biochemical substrates to assess both enzymatic activities and carbohydrate utilization patterns and other metabolic properties including sensitivity to Antibiotics. The cards were sealed after inoculation, incubated at 35–37 °C and interpreted with the Vitek® 2 software, as described in manufacturer's instructions [11].

2.4. Molecular confirmation of studied bacteria using polymerase chain reaction (PCR)

2.4.1. Genomic DNA extraction

Genomic DNA was extracted from overnight bacterial cultures using the Fla Pure Bacteria Genomic DNA Extraction Kit (Cat. No. DE703-50) according to the manufacturer's instructions, bacterial cells from 2 mL of overnight culture were harvested by centrifugation at 10,000

× g for 1 min. The bacterial pellet was lysed with the provided lysis buffer, then protein digests were performed with Proteinase K and protein was removed by precipitation. Genomic DNA was selectively bound to a silica membrane in high salt, washed to remove contaminants and eluted with low salt elution buffer [24].

DNA concentration and purity were determined spectrophotometrically using a Nano Drop spectrophotometer (Thermo Fisher Scientific, USA) by measuring absorbance at 260 and 280 nm. DNA integrity (no degradation) was verified by agarose gel electrophoresis (1.0%).

2.4.2. PCR amplification

PCR was performed to confirm bacterial identification by amplification of species-specific genes of *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella spp.* The primer sequences, target genes, and expected amplicon sizes are summarized in Table 1.

PCR reactions were carried out in a thermal cycler (Thermo Fisher Scientific, UK, in a final reaction volume of 25 µL. Each reaction mixture contained 12.5 µL of 2× PCR Master Mix (containing Taq DNA polymerase, MgCl₂, PCR buffer, and dNTPs), 1.0 µL of forward primer (10 µM), 1.0 µL of reverse primer (10 µM), 2.0 µL of template DNA, and 8.5 µL of nuclease-free water. The amplification program consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56–61 °C for 30 s (depending on the type of primer pairs), and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 3 min. Amplified PCR products were separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide and visualized under UV illumination. A 100-bp DNA ladder was used as the

molecular size marker to estimate PCR product sizes.

Table 1: represents Sequences of primers used in PCR assays for detection of specific genes in *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella spp.*

Bacteria	Target Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Product Size (bp)	Reference
<i>Staphylococcus aureus</i>	Nucleoside gene	GGATTGATGGTGATACGGTT	AGCCAAGCCTTGACGAACAAAGC	276	[52]
<i>Staphylococcus aureus</i>	26S rRNA	AACCTGCTATTAGGGAAGAACA	CCACCTTCTCCGGTTTGTACCC	750	[18]
<i>Listeria monocytogenes</i>	fljB gene	CCTAAGACGCCAATCGAA	AAGCGCTTGCAACGTGCTC	701	[45]
<i>Listeria monocytogenes</i>	26S rRNA gene	CTCCATAAGGTGAGCCT	CAGCMGCGGGGTAAATWC	938	[45]
<i>Salmonella</i>	invA	TATCGGCACGTTTGGCAA	TGCGACCGTCAAGGAACC	275	[55]
<i>Salmonella</i>	hlyA (SPI-2)	TTGAGCCATACGTAAGGAG	TCCATGAGGTGTTTACTCA	768	[34, 44]

3. Results and Discussion

3.1. Prevalence of Foodborne Pathogens in Analyzed Samples

This microbiological survey of 63 cheese samples using the standard bacterial culturing methods revealed significant contamination with three major pathogenic bacteria: *Listeria monocytogenes* was isolated from 3 of 63 samples (4.8% prevalence), *Staphylococcus aureus* was identified in 27 samples (42.85% prevalence) and *Salmonella spp.* was detected in 2 samples (3.17% prevalence). Overall, 32 of the 63 cheese samples (50.79%) were positive for at least one of the three target pathogens, whereas 31 samples (49.21%) were negative for all three pathogens.

The detection of *Staphylococcus aureus* as the most prevalent pathogen (42.85%) in the present study is consistent with global epidemiological studies reported for traditional dairy products. Close results have been documented in traditional Polish Koryciński cheeses produced from raw milk, in which staphylococci were detected in 68.9% of the samples [3]. Likewise, *S. aureus* was identified as the predominant foodborne pathogen in traditional white

cheese in Turkey [25]. The prevalence percent of *S. aureus* in traditional cheese increase to 96% in the study of [28], representing one of the highest prevalence rates recorded for specific bacterial pathogens in traditional cheeses.

Table 2 represent the prevalence percent of the bacteria used in this study

Bacterial Pathogen	Positive Samples	Prevalence (%)
<i>Listeria monocytogenes</i>	3	4.8%
<i>Staphylococcus aureus</i>	27	42.85%
<i>Salmonella spp.</i>	2	3.17%

The high prevalence of *S. aureus* observed in traditional cheese samples used in the current study is primarily attributed to the use of raw milk (without pasteurization) that obtained from animals with subclinical mastitis which represents an important primary source of contamination [7,20], and contaminated equipment, and poor sanitation practices further increase the risk of bacterial transmission during processing. Moreover, food handlers frequently serve as reservoirs of *S. aureus*, as the organism naturally colonizes the skin and nasal passages of healthy individuals. The inadequate refrigeration during the early stages of cheese manufacture may promote bacterial growth. Moreover, *S. aureus* is tolerant to relatively high salt concentrations and it is capable of forming biofilms on cheese surfaces, enabling its persistence in dairy processing environments [15].

The prevalence of *L. monocytogenes* observed in the present study (4.76%) was comparable to the prevalence reported in a systematic review of raw milk studies conducted in the United States between 2000 and 2019 (4.3%; 95% CI: 2.8–6.5%) [59]. similarly, a systematic review of soft cheeses conducted by [8] estimated the prevalence of *L. monocytogenes* at 2.4% (95% CI: 1.6–3.6%), which was substantially lower than the prevalence

observed in the traditional cheese samples analyzed in the current study. Whereas only 1.2% (95% CI: 0.0–4.2%) prevalence of *L. monocytogenes* contamination in mozzarella cheese reported in a comprehensive meta-analysis based on 16 studies, investigated by [9]. The results of this study is consistent with findings reported for other traditional cheeses from different regions. In Ethiopia, cottage cheese demonstrated an overall prevalence of 5.7% for *L. monocytogenes* [21]. However Portuguese cured raw milk cheeses from the Alentejo region showed *L. monocytogenes* contamination in 15.6% of samples [47]. Such variations in prevalence may be associated with differences in sample origin, production practices, sampling procedures, and storage temperature management. Other factors that increase contamination with *L. monocytogenes* include using raw milk, contaminated milking equipment, farm environments, and manufacturing conditions with limited standardization of hygienic practices, due to its psychotropic nature, allowing it surviving and multiplying at refrigeration temperatures [37], increasing the risk of contamination during storage and distribution. In addition, this pathogen is very resistant to common food preservation methods. Therefore, increased monitoring and control measures are required when *L. monocytogenes* are found in traditional cheeses.

The prevalence of *Salmonella* spp. in the cheese samples used in the present study was 3.17%, which is fall in the range of prevalence of *Salmonella* (95% CI: 2.0–6.2%) in raw milk cheeses in the United States [59] and close to the prevalence reported in white cheese samples from Turkey (11.9%) [35]. However, this prevalence was higher than the absence of *Salmonella* detection reported in Halloumi cheese in artisanal cheeses from the Ereğli province [35,14] and in raw-milk

Koryciński cheese from Poland [12]. While a considerably higher prevalence (9%) was reported in Mexican artisanal Cotija cheese samples [55]. Differences in *Salmonella* prevalence among studies may be associated with several factors, including the type of cheese, milk quality, production scale, and sanitation practices during processing. In traditional cheese production systems, contamination may occur through the use of raw milk obtained from infected animals, contaminated water, unsanitary equipment, or poor personal hygiene of food handlers. There may also be contamination of *Salmonella* in cheese products during transportation, storage or retail display. Besides, differences in detection rates could be due to differences in microbiological methodologies and enrichment procedures. The low number of bacteria or non-uniform distribution of the pathogen in cheese matrices could result in underestimation of contamination in some studies. The pH, salt concentration and competitive microflora found in some cheeses also might have an inhibitory effect on *Salmonella* survival and growth. The detection of *Salmonella* spp., even if at a low prevalence, is of great public health concern as this pathogen is one of the most important causes of gastroenteritis in the world caused by foodborne pathogens.

3.2. Bacterial identification using biochemical test (VITEK 2 system)

The isolates that presumptively identified as *Staphylococcus aureus* by culturing methods were further confirmed using the VITEK 2 GP Card system. The results showed that the isolates were identified as *S. aureus* with a good to acceptable level of confidence (90 – 99% probability for J-1, 1525-1 and C-1; and 85% probability for E-1). The analysis times were efficient, between 4.62 and 7.98 hours. The high level of confidence achieved in the present study is in line with recent studies [49, 36]

where VITEK 2 was applied in combination with molecular markers that based on PCR, for identification of dairy isolates. These results showed the reliability and sensitivity of the VITEK 2 system in the identification of *Staphylococcus* species from cheese.

The Vitek 2 system includes some biochemical tests that are highly metabolically consistent with the studied isolates. All of the *S. aureus* isolates gave positive results for Phosphatase, Alpha-Glucosidase and L-Pyrrolydonyl-Arylamidase. In contrast, they were all negative for Beta-Galactosidase and Beta-Glucuronidase. As per Carbohydrate Fermentation, production of Acid was found in all the carbohydrates, namely D-Mannose, D-Mannitol, D-Maltose, D-Glucose and Sucrose. But the isolates were unable to ferment D-Xylose, D-Raffinose. In Screening to Antibiotic Resistance, all isolates were able to grow on Polymyxin B and Bacitracin, and grew in 6.5% NaCl, which enable *S. aureus* to survive the high salt level of traditional cheeses [38].

Some minor phenotypic differences were observed, for example, isolate E-1 was positive for Cyclodextrin, while the other three were negative, indicating minor strain level diversity, typical of the variability found in the artisanal food environment where multiple staphylococcal strains are present [13]. Identification of *S. aureus* using VITEK 2 AST-GP79 card was also used by other studies [56, 36].

Furthermore, The 97% identification probability obtained using the VITEK® 2 automated identification system for *L. monocytogenes* isolates recovered from cheese samples in the current study indicates a high level of confidence in the species identification. This high probability suggests that the biochemical profiles of the isolates showed a strong match with the reference profiles available

in the VITEK® 2 database, supporting the reliability of the identification results. The VITEK 2 GP card showed positive esculin hydrolysis, catalase activity, arginine dihydrolase activity and ribose utilization and negative urease and mannitol fermentation. All *Listeria monocytogenes* isolates screened to antibiotic resistance are resistant to tetracycline, erythromycin and some fluoroquinolones. By contrast, most were susceptible to first line therapeutic drugs, especially ampicillin, and gentamicin. This result matching the system's database parameters and biochemical profiles that accomplished in a study conducted by [52]. When applying VITEK-2, over 40 tests, with sufficient resolving power to differentiate *L. monocytogenes* from morphologically similar species, like *L.innocua* and *L.welshimeri* [11].

Although VITEK® 2 successfully identified *L. monocytogenes* isolates recovered from positive cheese samples in the present study, these findings provide supportive evidence of its applicability to cheese matrices rather than demonstrating its overall efficacy for foodborne pathogen identification. However the accuracy of VITEK® 2 identification may be influenced by the physiological state of bacterial cells and other factors, such as the age of the bacterial culture, chemical treatment as well as growth and storage conditions [6]. Therefore, further evaluation of the system using molecular confirmation is becoming very important to increase the level of confidence in *L. monocytogenes* identification.

Regarding to *Salmonella* spp. isolate from a traditional cheese samples, it identified with the Vitek 2 automated system (bioMérieux, GN card) with 99% probability and given an "Excellent Identification" rating, which requiring 3.85 hours for analysis. A previous study also used VITEK® 2 system for the

identification of *Salmonella* isolates recovered from a wide range of food sources with identification accuracy of greater than 98% [29]. The biochemical characteristics of the isolates were typical of *Salmonella* spp. as all were positive for glucose fermentation, citrate utilization, lysine decarboxylase and indole production and negative for lactose fermentation, urease and indole production. Furthermore, most of the *Salmonella* isolates were susceptible to the cephalosporins and carbapenems, while they were resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole. The prevalence rate of this malty drug resistance (MDR) *Salmonella* is consistent with the recent results of a study showed high prevalence of *Salmonella* strains with resistance to penicillins and tetracyclines [29]. The ciprofloxacin and amikacin susceptibility of this isolate was different from other local studies, as isolates from outbreaks in Baghdad which were amikacin resistant, indicating regional differences in antimicrobial resistance [4]. Therefore, ongoing molecular surveillance and thorough genome sequencing of such isolates is crucial to understand the mechanisms of resistance and guide evidence-based treatment plans for *Salmonella* infections.

Use of automated systems VITEK in the present study offered several advantages over the conventional methods of identification (culturing), it reduced processing time, standardized interpretation and improved accuracy. Furthermore, VITEK 2 improve the reliability of bacterial identification, an important point for the food safety monitoring. Although all these benefits of the technique, some disadvantages were still reported such as identification of 1025 isolates of bacteria using the Vitek-2 system and finding error rates of 0.58% in genus level and 6.24% in species level [19] which may be due to some bacteria have

slow metabolism so they are susceptible to error of the Vitek-2 system; therefore, they should be confirmed again by other possible method [58]. Hence for accurate species identification it may be conform by more advanced techniques such as PCR.

4. Bacterial identification using Polymerase Chain Reaction (PCR)

4.1. DNA extraction

The genomic DNA was isolated successfully from the three target bacterial species (*Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* spp.) using a commercial DNA extraction kit (Cat. No. DE703-50/Jinsha Biology/China). The genomic DNA extracted was of good quality with A260/A280 ratio from 1.7 to 1.9, suitable for downstream molecular analyses. DNA integrity was confirmed by agarose gel electrophoresis where sharp and distinct bands without observing degradation or smearing, indicating the successful bacterial cell lysis and effective removal of protein and RNA contamination. The same DNA extraction kit used successfully for extraction DNA from bacteria in other studies [50,53]. Using this method is preferred as it recovered DNA in a faster and more reproducible way with fewer steps in the process, which minimized possible cross-contamination and loss of DNA during extraction.

The extraction profile for all three species was similar, suggesting that the lysis buffer used is effective against distinct cell wall structures Gram-positive (*L. monocytogenes* and *Staphylococcus aureus*), and gram-negative bacteria (*Salmonella* spp.), which present substantially different challenges during DNA extraction [41], indicating that the kit's lysis buffer are robust enough to overcome the varied cell wall challenges presented by these kinds of bacteria.

4.2. Application of PCR for bacterial identification

Identification of the studied bacteria that recovered from the traditional cheese and assessment of their virulence potential of was confirmed by PCR analysis, using primers targeting species specific genes for each studied bacteria. Presumptive *S. aureus* isolates were confirmed by the amplification of *16S rRNA* and *nuc* gene with expected size 750 bp and 276bp respectively (figure 1). These two DNA genes have shown to be effective for identification of *S. aureus* isolated from different food matrices. The *nuc* gene is a species specific marker has been reported to consistently amplify in all positive isolates of *S. aureus* [17]. This gene encodes a thermostable nuclease, and considered as one of the most important virulence factors and is a marker of enterotoxigenic potential, it has been highly used for the rapid molecular identification of *S. aureus* [48]. Previous studies have shown that nuc-positive *S. aureus* strains are often methicillin-resistant and have an increased capacity to form biofilms, as well as being enterotoxin producers [5]. The identification of both genetic markers simultaneously made identification more reliable and misclassification with other coagulase positive staphylococci less likely. Molecular confirmation is a special requirement because phenotypic methods can sometimes result in ambiguous identification because of biochemical variability between strains or unusual biochemical characteristics.

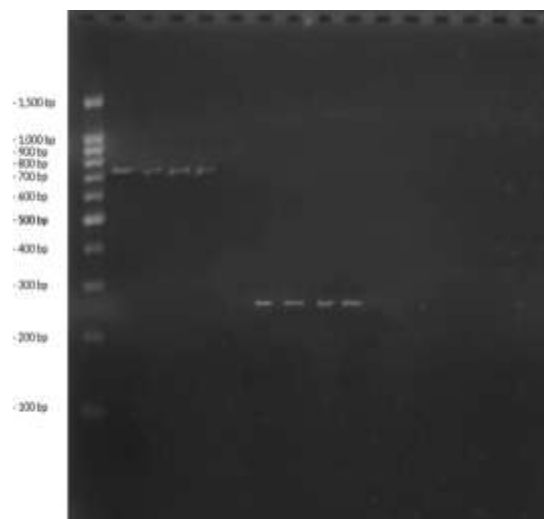


Figure 1: represent Gel electrophoresis analysis of PCR products specific for *Staphylococcus aureus*. Lanes 1–4 a specific amplicon band at 750bp of the 16S rRNA gene. Lane 5-8 a specific amplicon band at 276 bp. for Nuclease gene.

For *L. monocytogenes*, successful PCR amplification of both the *16S rRNA* and *hlyA* genes (figure 2) with expected size of 938bp and 701bp respectively, confirming species identification and virulence potential. These results are close to that of [10] and [50] who also employed PCR targeting *hlyA* and *16S rRNA* genes for *L. monocytogenes* identification in dairy products. Another study documented that all *L. monocytogenes* extracted from semi-hard cheese, harbored the *hlyA* gene indicating that this marker is ubiquitous among food-associated strains [22].

The *16S rRNA* gene is a highly conserved gene and used commonly for bacterial identification, while the hemolysin A (*hlyA*) gene is a major virulence factor for the pathogenesis of *L. monocytogenes* and is also a genus-specific marker. The *hlyA* gene encodes listeriolysin O, a pore-forming cytolysin which is essential for the intracellular life of *Listeria monocytogenes* and is involved in the disruption of the phagosomal membrane and escape of the pathogen into the host-cell cytoplasm. The

presence of *hlyA* is thus an important molecular marker for the virulence potential of *L. monocytogenes* isolates. Therefore, identification of *hlyA* is a useful tool in addition to species identification and can also help to characterize and assess the virulence potential of isolates and their potential for public health importance.

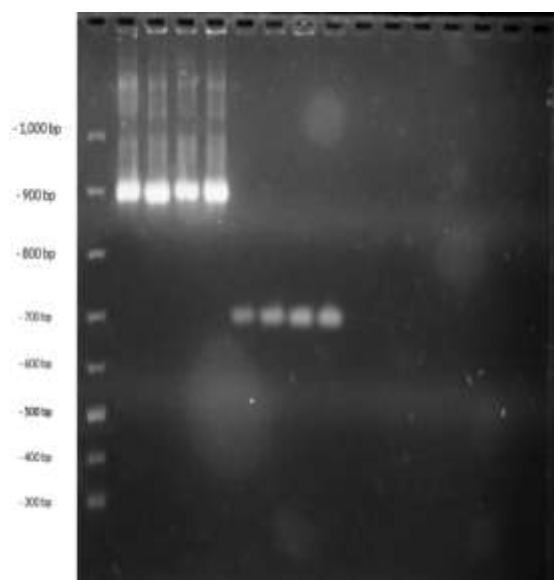


Figure 2: represent Gel electrophoresis analysis of PCR products specific for *Listeria monocytogenes*. Lanes 1–4 a specific amplicon band at 938 bp of the 16S rRNA gene. Lane 5-8 a specific amplicon band at 701 bp for *hlyA* gene.

The PCR assay also confirmed the presence of *Salmonella* spp. by amplification of the *invA* and *hlyA* gene with expected size of 275bp and 768bp respectively (figure 3). All the *Salmonella* isolates found to contain both genes which is a proof of the virulent of these strains and show that they have the genetic architecture required for intestinal invasion and systemic infection. These results are similar to those in previous research, in which 100% of *Salmonella* isolates obtained from dairy products were positive for *invA* and *hlyA* [29]. *InvA* gene detection has also been found reliable in other studies, and this gene has been found in

100% of *Salmonella* serovars [1, 60]. The gene *invA* is one of the most conserved genes among the pathogenic serovars of *Salmonella* spp. and encodes an essential protein for invasion of epithelial cells that is essential for intracellular survival, making it an ideal molecular target for *Salmonella* detection, hence it considered the "international gold-standard". Similarly, the *hlyA* gene is a hub gene that is involved in the regulation of invasion-associated genes in *Salmonella* Pathogenicity Island 1 (SPI-1). The presence of both genes is thus good indicator of pathogenic and invasive *Salmonella* strains.

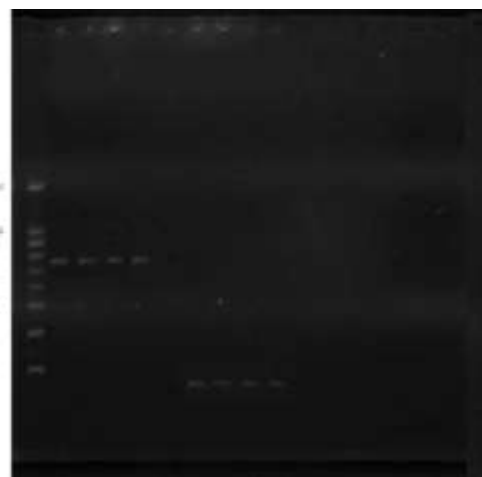


Figure 3: represent Gel electrophoresis analysis of PCR products specific for *Staphylococcus aureus*. Lanes 1–4 a specific amplicon band at 750bp for the *hlyA* (SPI-1) gene. Lane 5-8 a specific amplicon band at 275 bp. for *invA* gene.

The success of PCR-based detection in this study depend critically on DNA extraction efficiency, primer design, number of copies of target genes per bacterial cells and optimization of thermal cycling conditions. It was crucial to select primers for conserved areas that were species specific. The reliability of 16S rRNA gene-based PCR has been well-established; with studies demonstrating its utility in confirming bacterial identity across diverse food matrices [31].

The presence of virulence associated genes such as *hlyA*, *nuc*, *invA* and *hlyA* in isolates indicates that these isolates possess pathogenic characteristics that may increase the risk of foodborne illness among consumers. The use of molecular diagnostics in combination with standard microbiological tools can enhance food safety surveillance programs and help prevent foodborne disease outbreaks.

5. Conclusions

This study demonstrated that traditional cheese samples sold in Sulaimani market represent a potential microbiological safety concern due to the detection of 3 kinds of foodborne pathogenic bacteria. *Staphylococcus aureus* was the most common pathogen followed by *Listeria monocytogenes* and *Salmonella spp.* The use of conventional culture-based methods for recovery of bacterial isolates, VITEK® 2 identification system as rapid technique based on biochemical profiles, and polymerase chain reaction (PCR) that supported the identification of target pathogens and provided additional molecular evidence for the presence of specific virulence-associated genes. All the techniques provided a reliable and complementary approach for the identification of bacterial pathogens in the studied cheese samples. . Regular bacterial detection of traditionally produced cheese and the application of rapid identification methods are recommended to enhance food safety surveillance and protect consumers from potential foodborne infections.

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