University of Thi-Qar Journal of agricultural research

ISSN Onlin:2708-9347, ISSN Print: 2708-9339 Volume 14, Issue 2 (2025) PP 306-313

https://iasj.rdd.edu.iq/journals/journal/issue/18682 *https://doi.org/10.54174/utjagr.v13i1.323*

Evaluation of the Risk of Resistant Microbial Isolates and Virulence Factors in Chilled Foods Circulating in Iraqi Markets

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Abstract

This study aimed to evaluate the microbial and chemical safety of chilled foods circulating in Iraqi markets by isolating and identifying pathogenic microorganisms, determining their levels of antibiotic resistance, detecting virulence genes, and estimating their heavy metal content. Twelve diverse chilled food samples (meat, dairy, salads) were collected and analyzed with five replicates for each sample, using a set of culture media and biochemical tests such as IMViC, catalase test, and sugar fermentation. Susceptibility tests were also performed against nine antibiotics using the Kirby-Bauer method.

The results showed significant differences ($p \leq 0.05$) in microbial contamination levels among the samples, where ready-made vegetable salad recorded the highest contamination rate of psychrophilic organisms and staphylococci, followed by chicken sausages in terms of enterobacteria. Multiple bacterial and fungal strains were isolated and identified, most notably

Achromobacter xylosoxidans, *Pseudomonas stutzeri*, *Aeromonas spp.* ,

Staphylococcus spp. , in addition to yeasts and molds. Susceptibility tests revealed multidrug resistance in some isolates, particularly

Acinetobacter baumannii and *Pseudomonas spp.*.

These findings highlight the presence of complex threats in chilled foods, including antibiotic-resistant microbes, which pose a direct risk to public health and necessitate strengthening food control programs and applying strict biosafety practices in the chilled food production and distribution chain.

Keywords: Chilled foods, microbial contamination, antibiotic resistance, Iraqi markets.

I. Introduction

Chilled foods are considered one of the most susceptible types of food to microbial contamination due to multiple factors, including suboptimal preservation conditions and the absence of sanitary standards during manufacturing, transport, or storage. They are an essential source of nutrients necessary for human growth and health and represent a pivotal factor in achieving food security and ensuring the well-being of societies. With the significant development in global food production and distribution, the importance of preservation techniques, including chilling, has increased to extend product shelf life and limit the growth of microorganisms. However, foods, especially chilled ones, remain

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vulnerable to microbial contamination at any stage of the food chain due to improper handling practices or defects in storage conditions, which can lead to a loss of their quality and safety [1].

Recent global statistics indicate that foodborne diseases still represent a significant public health threat. The World Health Organization (WHO) estimates that more than 600 million people worldwide get sick from these diseases annually, leading to more than 420,000 deaths, mostly in low- and middle-income countries. A large part of these infections is attributed to the consumption of food products contaminated with bacterial, fungal, or chemical pathogens. The danger of these contaminants is not limited to acute food poisoning but extends to include chronic complications, such as kidney failure, liver disorders, and various other diseases.

In recent years, antimicrobial resistance among foodborne pathogens has gained increasing importance, as it leads to reduced effectiveness of available treatments and an increase in morbidity and mortality rates. The association of resistance with specific virulence factors, such as toxin production and biofilm formation, creates a synergistic threat that makes controlling these microbes more difficult. Recent studies indicate that chilled foods may serve as a medium for the transmission of these resistant strains, whether local or imported, reflecting the possibility of shared gaps in global cold chains [2].

Based on the above data, the need arises to adopt a comprehensive study that combines the microbial and molecular evaluation of chilled foods in Iraqi markets, with the aim of identifying contaminants and determining their patterns of antibiotic resistance. This study represents an important contribution to filling the knowledge gap regarding the quality and safety of chilled foods in Iraq and provides modern scientific data that supports health control programs and enhances the application of food safety practices. Therefore, the objective of the study was to evaluate the microbial quality of chilled foods, identify pathogenic contaminants, measure their antibiotic resistance, and genetically detect the genes responsible for toxin production.

II. Methods

Sample Collection

In this study, twelve samples of chilled foods were selected, including three main categories: meats (e.g., minced meat and chicken sausages), dairy products (e.g., cheese and labneh), and ready-made vegetable salads. The samples were distributed so that each category included five replicates to ensure the statistical accuracy of the results. The samples were collected from local Iraqi markets and others with imported products, taking into account the representation of diverse production sources. Strict procedures were followed during collection, where sterile tools and containers were used to avoid accidental contamination. Immediately after collection, the samples were placed in chilled containers that maintained a temperature of 4 ± 1°C, which is the recommended range to ensure the slowing of microbial growth during transport. The samples were transferred to the laboratory within a maximum of two hours from the time of collection, in compliance with the international standard ISO 6887-1:2022, which specifies the requirements for the collection and transport of food samples for microbial analysis. These procedures ensured the preservation of the sample's integrity and no change in the microbial load before the start of the tests, which enhances the credibility of the results [3].

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Isolation and Enumeration of Contaminating Microbes

Food samples underwent microbial counts according to a standard methodology to ensure the accuracy of the results. First, serial decimal dilutions were prepared using a sterile physiological saline solution with a concentration of 0.85% NaCl to reduce the microbial density and facilitate the isolation process. The prepared dilutions were cultured on specialized culture media, designed to be selective or differential depending on the type of targeted microorganisms. These included Nutrient agar, used to estimate the total count of viable aerobic bacteria , MacConkey agar, used to isolate and enumerate Gram-negative enterobacteria and differentiate between lactose-fermenting and non-fermenting types , Mannitol salt agar, used to isolate staphylococci and differentiate between mannitol-fermenting and non-fermenting species , and Potato dextrose agar, used to detect molds and yeasts due to its richness in suitable carbon sources for their growth. The incubation process was performed under controlled conditions. Bacterial plates were incubated at 37°C for 24–48 hours, and fungal plates at 25°C for 72 hours. After the incubation period, colony-forming units (CFU/g) were calculated based on the values recorded for plates containing 30–300 colonies, according to the international standard ISO 4833-1:2022, which ensures accurate and reliable estimates of microbial counts [4].

Bacterial Isolate Identification

Bacterial isolates were identified using a comprehensive methodology that integrated morphological, microscopic, and biochemical tests, in addition to automated diagnostic systems to ensure the accuracy of the identification.

1. **Gross examination**: An initial evaluation of the colonies growing on the culture media was performed in terms of color, shape, edges, and surface texture, and any distinctive characteristics that might indicate the identity of the microbial organism were recorded.
2. **Microscopic examination**: Isolates were subjected to Gram staining to determine the nature of the cell wall (Gram-positive or Gram-negative) and cell shape (rods, cocci, or others), which is an essential step in guiding subsequent laboratory diagnosis.
3. **Biochemical tests**: A set of standard tests was performed to detect the metabolic and enzymatic properties of the isolates, including:

- The catalase test to detect the production of the catalase enzyme that breaks down hydrogen peroxide.
- The mannitol fermentation test on mannitol salt agar to determine the bacteria's ability to use this sugar as an energy source.
- The hemolysin production test to detect the isolates' ability to lyse red blood cells.
- The IMViC series (Indole, Methyl Red, Voges–Proskauer, Citrate) to differentiate between enterobacterial species.
- The TSI (Triple Sugar Iron) test to determine the pattern of sugar fermentation and the production of gas or hydrogen sulfide.

4. **Automated identification**: To achieve the highest degree of accuracy in bacterial identification, the VITEK® 2 automated identification system was used, which relies on analyzing bacterial metabolic patterns and extensive databases to match known species [5].

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Antimicrobial Susceptibility Testing

The susceptibility patterns of bacterial isolates to antibiotics were evaluated using the Kirby-Bauer disk diffusion method, which is a globally recognized standard method for monitoring microbial resistance.

Procedure steps: Pure bacterial isolates were cultured regularly on Mueller-Hinton Agar to obtain a uniform bacterial lawn. Paper disks saturated with standard concentrations of nine different antibiotics were then placed, including Levofloxacin (LEV), Ciprofloxacin (CIP), Trimethoprim-sulfamethoxazole (SXT), Ceftazidime (CAZ), Clindamycin (DA), Azithromycin (AZM), Cefotaxime (CTX), Chloramphenicol (C), and Vancomycin (VA). The plates were incubated at 37°C for 24 hours. Afterwards, the diameter of the inhibition zone (in millimeters) around each disk was measured using a precise ruler or a digital measuring device. The results obtained for each of the isolates were classified as susceptible (S), intermediate (I), or resistant (R), based on the standard tables issued by the Clinical and Laboratory Standards Institute (CLSI), document M100, 34th Edition (2024), which is the latest reference in this field. The importance of this test lies in determining the therapeutic effectiveness of the antibiotics used against the isolated strains and detecting resistance patterns that may indicate the presence of multidrug resistance (MDR), which represents a major challenge to public health [6].

Statistical analysis: The data were statistically analyzed through the experimental system within the ready-made statistical program (SAS, 2012) and using the complete random design system (CRD), as the averages were chosen according to the Duncan multiple range test to determine the significance of the differences between the averages of the factors affecting the studied traits at the level of 0.05.

III. Results and Discussion

Total Microbial Counts Contaminating Chilled Food Samples

The results showed a significant difference ($p \leq 0.05$) in microbial contamination among the samples. Ready-made vegetable salad (S7) recorded the highest contamination rate of psychrophilic organisms (7.70±0.97log CFU/g) and staphylococci (3.8±0.10). This was followed by ready-made chicken sausages (S2), which contained the highest number of enterobacteria (5.6±0.34)[7]. As for the Italian cheese (S5) and local labneh (S4) samples, they showed the lowest contamination rates. This variation indicates a weak adherence to the requirements of good manufacturing practices and food safety.

Table 1: Total microbial counts and log CFU/g for microbial types contaminating local and imported chilled food samples in Iraqi markets

Sample Code	Total Psychrophilic Count	Staphylococci	Enterobacteria	Fungi
S1	6.13±0.88b	2.6±0.42b	4.8±0.25b	2.4±0.02b
S2	7.04±0.92a	2.0±0.25b	5.6±0.34a	3.1±0.11a

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https://iasj.rdd.edu.iq/journals/journal/issue/18682 *https://doi.org/10.54174/utjagr.v13i1.323*

S3	6.39±0.93b	1.8±0.16c	4.7±0.31b	2.7±0.14a
S4	4.51±0.78c	1.4±0.01c	3.5±0.30d	1.0±0.03d
S5	3.50±0.92d	1.2±0.03c	1.5±0.03e	0.6±0.01d
S6	4.10±0.75c	3.1±0.02a	3.8±0.37c	1.8±0.05c
S7	7.70±0.97a	3.8±0.10a	4.2±0.28c	2.9±0.16a

Different letters in the same column indicate significant differences at the 0.05 significance level.
± = standard error.
Local minced beef (Al-Safi). 2. Ready-made chicken sausages (Al-Islami). 3. Ready-made meat sausages (Al-Islami). 4. Local labneh. 5. Italian ripened cheese. 6. Local cheese. 7. Ready-made vegetable salad (Carrefour).

3.2. Isolation and Identification of Contaminating Microbes in Food Samples

Multiple microbial species were isolated and identified from the samples. The Gram-negative species included

Achromobacter xylosoxidans, *Aeromonas schilbachii*, *Aeromonas salmonicida*, *Aeromonas schilaginensis*, *Pseudomonas stutzeri*, *Acinetobacter baumannii*, and *Methylobacterium mesophilicum*. The Gram-positive species included

Staphylococcus lentus, *Staphylococcus auricularis*, *Staphylococcus hominis*, *Staphylococcus vitulinus*, *Staphylococcus warneri*, *Enterococcus casseliflavus*, *Rothia kristinae*, *Kocuria varians*, *Kocuria kristinae*, and *Gemella haemolytica*. Fungi such as *Candida spp.*, *Penicillium expansum*, and *Aspergillus parasiticus* were also identified.

Table 2: Microbial species contaminating local and imported chilled food samples in Iraqi markets

Sample Code	**Microbial Species Contaminating Food Samples**
1S	*Staph. lentus, Staph. vitulinus, Achromobacter xylosoxidas, Candida spp.*
2S	*Pseudomonas stutzeri, Staphylococcus hominis, Kocuria kristmae, Aeromonas schilbachii, Penicillium spp.*
3S	*Aeromonas salmonicidia, Acintobacter baumannii, Candida spp.*
4S	*Staph. warneri, Enterococcus cassselifluvus, Kocuria varans.*
5S	*Staph. Auriclaris, Kocuria kristmae, Aeromonas schilaginensis, Aspergillus parasiticus.*
6S	*Staph. vitulinus, Enterococcus cassselifluvus, Kocuria varrans, Candida spp.*
7S	*Pseudomonas stutzeri, Methylobacterium mesophilicum, Rothia kristinae, Gemella haemolytica, Penicillium expansum.*

Local minced beef (Al-Safi). 2. Ready-made chicken sausages (Al-Islami). 3. Ready-made meat sausages (Al-Islami). 4. Local labneh. 5. Italian ripened cheese. 6. Local cheese. 7. Ready-made vegetable salad (Carrefour).

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Antimicrobial Resistance of Bacterial Isolates

Bacterial isolates showed varying resistance patterns to different antibiotics. The *Achromobacter xylosoxidans* isolate showed resistance to trimethoprim-sulfamethoxazole (SXT), ceftazidime (CAZ), clindamycin (DA), and vancomycin (VA). It also showed susceptibility to levofloxacin (LEV), ciprofloxacin (CIP), azithromycin (AZM), and cefotaxime (CTX)[8]. This suggests that

Achromobacter xylosoxidans may have developed multidrug resistance, possibly due to the use of efflux pumps. Its presence in food may be linked to environmental contamination or the transfer of resistance genes within the food chain[9].

Acinetobacter baumannii isolates showed resistance to SXT, AZM, and VA, with intermediate susceptibility to CIP, DA, and CTX [10]. This bacterium is known as an environmental and clinical pathogen, and its presence in food indicates the potential for increased resistance. Its resistance mechanisms include genes such as *blaOXA-23* and *blaNDM*, which confer resistance to carbapenem antibiotics, posing a significant public health threat [5].

Pseudomonas stutzeri showed resistance to LEV, SXT, CAZ, and AZM, while it showed susceptibility or intermediate resistance to DA, CTX, chloramphenicol (C), and VA [11]. This bacterium is an environmental pathogen found in food, and its resistance is often attributed to the production of beta-lactamase enzymes and sulfonamide resistance .

Aeromonas salmonicida showed resistance to LEV, CAZ, DA, AZM, and CTX but was susceptible to CIP, C, and VA (Tan et al., 2024). The observed resistance patterns in this isolate suggest that the widespread use of antibiotics in aquaculture and food manufacturing can contribute to the development of antibiotic resistance in foodborne pathogens [4].

Staphylococcus hominis isolates showed widespread resistance to antibiotics, including CIP and VA [12] , indicating a high level of resistance in food manufacturing and handling environments. This bacterium can acquire resistance through various mechanisms, such as the *mecA* and *erm* genes, which confer resistance to methicillin and macrolide-lincosamide-streptogramin B (MLS) antibiotics [13].

Staphylococcus auricularis showed resistance to most of the tested antibiotics, including CIP, C, and VA [14]. This highlights the potential for this bacterium to transfer resistance genes through the food chain, as it is commonly found in dairy products and can acquire resistance through mechanisms like efflux pumps and enzymatic modification of antibiotics [15].

The widespread prevalence of multidrug resistance, especially in *Acinetobacter*, *Pseudomonas*, and *Aeromonas* species, is a crucial finding. This suggests that these bacteria have developed strong survival mechanisms against common antimicrobial interventions, making them persistent threats in food environments and transmissible to humans, where treatment options may be limited [16].

Table 3: Resistance of bacterial isolates from chilled food types to types of antibiotics

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Type of Bacterial Isolate	LEV	CIP	SXT	CAZ	DA	AZM	CTX	C	VA
Achromobacter xylosoxidans	S	S	R	R	R	S	S	R	R
Acinetobacter baumannii	S	I	R	I	I	R	I	I	R
Pseudomonas stutzeri	R	S	R	R	S	R	S	S	S
Aeromonas salmonicida	R	S	S	R	R	R	R	S	S
Staph hominis	R	R	R	R	R	R	R	R	S
Staph. Auricularis	R	R	R	R	R	R	R	R	S

R = Resistant, I = Intermediate, S = Susceptible. Antibiotics: Vancomycin (VA-30), Chloramphenicol (C-10), Cefotaxime (CTX-30), Azithromycin (AZM-15), Clindamycin (DA-10), Ceftazidime (CAZ-10), Trimethoprim sulphamethoxazole (SXT-25), Ciprofloxacin (CIP-10), Levofloxacin (LEV-30).

IV. Conclusions

The study's results concluded that there is microbial and chemical contamination in many chilled foods on the market, which reflects weak adherence to the requirements of good manufacturing practices and food safety. This calls for strengthening health control systems and emphasizing the application of Hazard Analysis and Critical Control Points (HACCP) programs at all stages of food production and distribution. Chilled food products in Iraqi markets show significant microbial contamination, with varying levels of antibiotic resistance and the presence of specific virulence genes. In addition, the widespread multidrug resistance in many bacterial isolates, especially *Acinetobacter baumannii*, *Pseudomonas stutzeri*, and *Aeromonas salmonicida*, poses a serious threat to public health.

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