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Microbial risk assessment of dairy products from retail marketplaces in Basrah province, Iraq

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Abstract

Background: Milk-borne bacteria cause degradation of milk products and constitute a significant risk to public health.
Aim: The objectives of the present study are to determine the microbiological quality of dairy products and to investigate pathogenic microorganisms.
Methods: A total of 60 samples of raw milk, homemade cheese, and yogurt were randomly selected from different retail marketplaces in Basrah. The bacteriological and biochemical tests were utilized to identify the pathogens in dairy samples, as well as the molecular technique was used as an accurate diagnostic test.
Results: The prevalence of contamination of milk products with various isolates was estimated as 50% (95% Cl: 36.8–63.2). The mean of total bacteria count for cheese was 7.29 ± 2.70, raw milk 4.62 ± 2.86, and yogurt 2.87 ± 1.05, with a significant *p*-value ($p = 0.001$). The mean count of aerobic spore-forming (ASF) contaminated raw milk was analyzed as 3.77 ± 1.18 and less contamination detected in the yogurt samples with mean of ASF was estimated as 2.52 ± 1.47 SD log 10 CFU/ml. A range of important microorganisms to human health were identified by employing the VITEK_2 system and sequencing 16S rDNA gene, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aerogenosa*, and *Bacillus cereus*.
Conclusion: The study indicates that there is a high level of bacterial contamination in dairy products with different bacteria species, which is medically important. Therefore, food safety management must be implemented to reduce biological risks carried by dairy products and ensure healthy food for consumers.
Keywords: Food safety, Milk products, Microorganism identification.

Introduction

Dairy products remain a priority in nutritious food and are excellent sources for human health (Górska-Warsewicz *et al*., 2019). Milk may serve as an ideal substrate for the growth and survival of a variety of microorganisms, constituting a threat to public health (Quigley *et al*., 2013). Microorganisms present in milk products can influence the flavor, taste, and texture of the finished forms of foods. Since milk is principally meant for human consumption, it should be free of all hazardous microbes (Garedew *et al*., 2012). Microbial contamination in milk may cause milk-borne diseases while others are known to cause milk spoilage or decomposition. Based on statistical evidence, dairy products contaminated with microorganisms contribute to 4% of all food-borne diseases, with the economic burden estimated to be US $95.2 billion in the Middle East and developing countries (Jaffee *et al*., 2019; Kapoor *et al*., 2023). In addition, foodborne illnesses are responsible for 33 million losses of the healthy life years (DALYs), while indirect economic losses resulting from medical expenses for treatment were estimated at US $110 billion (FAO, 2022). The most commonly manifested symptoms of milk-borne bacterial diseases in patients include fever, headaches, abdominal pain, diarrhea, and in-progression illness leading to kidney failure.

Sources of microbial contamination in milk include primary microbial contamination from the infected or carrier animals. Across the milk value chain, several variables contribute to microbial contamination comprising milk handlers, unsanitary utensils, and milking equipment (Berhe *et al*, 2020; Deddefo *et al*., 2023). Manure and soil are other sources of contamination milk with certain pathogenic bacteria, such as Coliform and *Enterobacteriaceae* (Manyi-Loh *et al*., 2016). Although pasteurization is still one of the most effective antibacterial methods for limiting bacteria growth in milk, lacking hygiene measurements throughout the manufacturing process leads to

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contamination with ubiquitous and biofilm bacteria (Calahorrano-Moreno *et al*., 2022). The quality of milk, therefore, is determined by its composition and overall hygiene standard measures.

Dairy products are relatively perishable commodities that are readily contaminated by sporeformers and a range of aerobic bacteria. The properties of sporulation bacteria, including *Bacillus* spp., are resilient to pasteurization temperature, radiation, and chemicals (Grutsch *et al*., 2018). The morphological structure of bacterium spores is composed of three or two distinctive layers, primarily the peptidoglycan layer that coats the spores' exterior and the small acid-soluble proteins layer that coats the chromosomes and DNA (Setlow; 1994; Stelder *et al*., 2017). Accordingly, spores can thoroughly survive in heat processing or pasteurization temperature. Raw milk in bull tanks is probably contaminated with spore-forming bacteria from a wide range of sources, including soil, animal waste, dirty milking equipment, teats, and bedding materials (Heyndrickx, 2011). Other bacteria species that frequently contaminate dairy products include *Pseudomonas*, *Escherichia coli*, and *Klebsiella pneumonia.* These microorganisms can survive within ambient or cool temperatures, and it is accountable for the spoilage of dairy products and monetary burden (Samaržija *et al*., 2012; El-Sayed *et al*., 2022).

Food-borne diseases are caused by a variety of pathogenic microorganisms from the consumption of contaminated milk products and other food items. Typhoid fever, brucellosis, and other bacterial diseases are the most common food-borne diseases reported in the Middle East and North Africa (Havelaar *et al*., 2015). For instance, the prevalence of *Brucella spp.* in milk products of buffalo and cows in the Middle Eastern countries was assessed as 29% (Abedi *et al*., 2020). Importantly, the potential growth of the dairy market in Iraq is driven by the increasing growth of populations and rising demands for consuming dairy products, especially among young people (USAID, 2006). Besides, there is insufficient cooling and hygienic handling of milk during transportation from farms to manufacturers, according to the report published by the International Trade Centre (ITC, 2022). Few studies, however, were carried out to evaluate or identify the risk of bacterial contamination in milk products in Basrah (Abbas and Al-Deewan, 2009; Abbas and Talei, 2010), which drastically poses a threat to community health due to food poisoning (Jaber *et al*., 2021). Therefore, this study aimed to assess the microbiological quality and to identify pathogenic microorganisms in dairy products from local marketplaces in Basrah.

Material and Methods

Samples collection

A total of sixty dairy products (20 raw milk, 20 homemade cheese, and 20 yogurt) were collected randomly from different marketplaces in Basrah province. The sampling was performed from March to July 2022, where the marketplaces were visited three times in a week for this purpose. The samples were kept in sterilized containers and directly transported to the microbiological lab located at the College of Veterinary Medicine (sample collection and transfer took around three hours). At the laboratory, the solid cheese samples (weighted 10 g) were rehydrated by dissolving them in 90 ml with sterile saline and then placed in a polyethylene bag which was shaken for around 5 minutes to be homogenized (Marshall, 1992). All collected samples were kept at 4°C in a refrigerator for the next microbiological tests.

Bacteriological and biochemical tests for identification bacteria

To eliminate all other types of bacteria excluding spore-forming bacteria, the cheese suspension samples (5 ml) were transferred into glass tubes and placed into the water bath (80°C) for 30 minutes (Difco, 1984). The quantity of bacteria in each sample was determined based on the standard plate count procedure (Brown and Smith, 2017). Serial dilution was prepared and then cultivated on the nutrient agars, subsequently incubated in the culture at 37°C for 48 hours. The number of visible colonies was counted and only numbers between 30 and 300 CFUs were considered as valid numbers.

Other selective media were utilized to identify the putative bacteria species using blood agar, MacConkey agar, and Eosin Methylene blue. Different colonies based on their morphological characterizations were determined and after that underwent subculture cultivation on nutrient agars. The single isolated colonies were subjected to Gram stain and biochemical reactions involving oxidase, catalase, and indole. To emphasize spore-forming bacteria, colonies were stained with malachite green dye and inspected under a light microscope. The motile from non-motile bacteria were observed by adopting the hanging drop method. Briefly, a sample of culture was suspended in saline and placed on a concave well in the slide, which was covered after that and examined under a light microscope. However, the purified culture was also stored on brain heart infusion for the purpose conducting of the VITEK-2 system and molecular tools (PCR), as the next step.

Identification of isolates bacteria by VITEK-2 system

The isolates of the purified specimens were subjected to the VITEK-2 system (version: 07.01), which basically offers an automated computer-based technique through quantifying the light attenuation associated with each biochemical reaction in VITEK cards. This scientific appliance is capable of identifying 95.8% of Gram-negative bacteria and 89.2% of Gram-positive bacteria (Nimer *et al*., 2016).

Molecular diagnosis of the isolates

A genomic DNA bacteria kit (Geneaid/Korea) was used to extract the DNA of the isolates forming spores. The results, in which the DNA is visible as bands under

UV light, were initially identified by electrophoresis on 0.8% Agarose (Sambrook and Russell, 2001). The PCR-amplification of the target gene 16S rDNA is used to detect *Bacillus* spp, with forward primer F-5'-AGAGTTTG ATCCTGGC-3' and reveres primer R- 5'-GGTTACCT TGTTACGACTT-3'). The PCR program basically includes an initial denaturation at 94°C for 5 minutes, then 37 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 0.5 minute, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes (Eden *et al*., 1991). Sequencing of the gene was performed by the National Instrumentation Centre for Environmental Management (NICEM) and the algorithm programme (BLAST) was conducted to compare the results with the database of sequences.

Statistical analysis

The data were analyzed by the statistics software (STATA Corp. 14.2, USA). Analysis of variance (One-Way ANOVA) was conducted to compare the means of bacteria count in the milk samples. The *p*-value for ≤0.05 is considered significant between variables. The identified bacteria species/strains from laboratory test results are illustrated in tables.

Ethical approval

Not needed for this study.

Results

Proportion and enumeration of total bacteria count (TBC)

Half of the dairy products samples (mean: 50%, 95% Cl: 36.8–63.2) were found to be positive with different bacteria isolates. The results of the TBC and aerobic spore-forming (ASF) for 30 different positive samples are illustrated in Table 1. The highest mean of TBC was observed in cheese (7.29 ± 2.70), followed by raw milk 4.62 ± 2.86 and yogurt 2.87 ± 1.05, with statistically significant ($p = 0.001$). The mean count of ASF in raw milk samples was found to be 3.77 ± 1.18, while less contamination was presented in the yogurt samples with a mean analyzed of 2.52 ± 1.47 SD log 10 CFU/ml, as well as significant differences reported ($p = 0.04$). In addition, the isolates especially the spore-forming bacteria were found positive for staining (acquired green color) and being motile. The visible colonies on selective media showed diversity in colors such as golden, metallic shiny, and bluish–green. The shapes of stained isolates were rod and spherical.

Biochemical identification of bacteria using the VITEK-2 system

A total of 15 bacterial isolates were biochemically determined using the VITEK-2 system (Table 2). The identified bacteria were *E. coli*, *Staphylococcus aureus, Pseudomonas aerogenosa,* and *Leuconostoc ssp. Staphylococcus* spp seems to be the most detected species in the dairy samples. *Escherichia coli* was detected in two samples (milk and cheese) and the bacterium (*P. aerogenosa*) presented in one sample (cheese).

Molecular detection of Bacillus by amplifying the 16S rDNA

The PCR was performed on only 10 samples. The result was detected by electrophoresis on 0.8 % Agarose and showed under UV light, then an extracted DNA from each isolate was subjected to PCR for amplifying 16S rDNA, as shown in Figure (1). The amplicon bands of the gene were characterized by 1,500 bp compared to the standard molecular DNA Ladder (2,000bp), and that confirmed the specific region for the bacterium strain of *Bacillus* spp.

Sequencing 16S rDNA and identification of ASF isolates

The closet strains with the highest percentage of identity in the forward and the reverse were selected and listed in Table 3. The sequences of 16S rDNA recognized 10 strains/substrains of *Bacillus* spp. Two samples were identified as *Bacillus cereus*, which is an important food-borne pathogen and always being harmful to human health. Other non-pathogenic strains of *Bacillus* including *Bacillus amyloliquefaciens* and *Bacillus subtilis* were identified.

Discussion

The present study estimated the TBC and ASF and different psychrotrophic bacteria were identified using both traditional and state-of-the-art laboratory methods. A variety number of psychrotrophic pathogenic bacteria were detected in the present study, which are able to grow and survive in low temperatures (2°C–7°C). These microorganisms, in particular the aerobic Gram-positive bacteria, can alter milk parameters and textures, as well as their tastes to unpleasant or formed rancidity due to proteolytic enzymes (García-Cano *et al*., 2019). The source of contamination of dairy products with microorganisms arises from the beginning of harvested milk at farms or maybe throughout storage, transportation, and preservation of milk production (Sotohy *et al*., 2022). Fresh dairy

Table 1. TBC and Aerobic spore-forming count (ASF) were observed in growth colonies.

Type of samples	TBC			ASF		
	Mean ± SD	log CFU/ml	*p*-value	Mean ± SD	log CFU/ml	*p*-value
Raw milk	4.62 ± 2.86	10^5–10^6		3.77 ± 1.18	10^3–10^4	
Cheese	7.29 ± 2.70	10^5–10^6	0.001	3.99 ± 1.42	10^3–10^4	0.04
Yogurt	2.87 ± 1.05	10^4–10^5		2.52 ± 1.47	10^3	

Table 2. Type of microorganisms identified from purified colonies using VITEK-2.

Sample number	Sources of sample	Identified bacterial species	Probability
1	Raw milk	*Staphylococcus lugdunensis*	85%
2	Raw milk	*Staphylococcus aureus*	90%
3	Raw milk	*Staphylococcus pseudointermedius*	87%
4	Raw milk	*Escherichia coli*	90%
5	Raw milk	*Staphylococcus pseudointermedius*	87%
6	Cheese	*Escherichia coli*	89%
7	Cheese	*Leuconostoc mesenteroides* ssp dextranicum	94%
8	Cheese	*Staphylococcus vitulinus*	93%
9	Cheese	*Pseudomonas aerogenosa*	89%
10	Cheese	*Staphylococcus aureus*	90%
11	Yogurt	*Staphylococcus lentus*	95%
12	Yogurt	*Staphylococcus lugdunesis*	85%
13	Yogurt	*Leuconostoc mesenteroides* ssp cremoris	90%
14	Yogurt	*Staphylococcus pseudointermedius*	95%
15	Yogurt	*Leuconostoc mesenteroides* ssp cremoris	89%

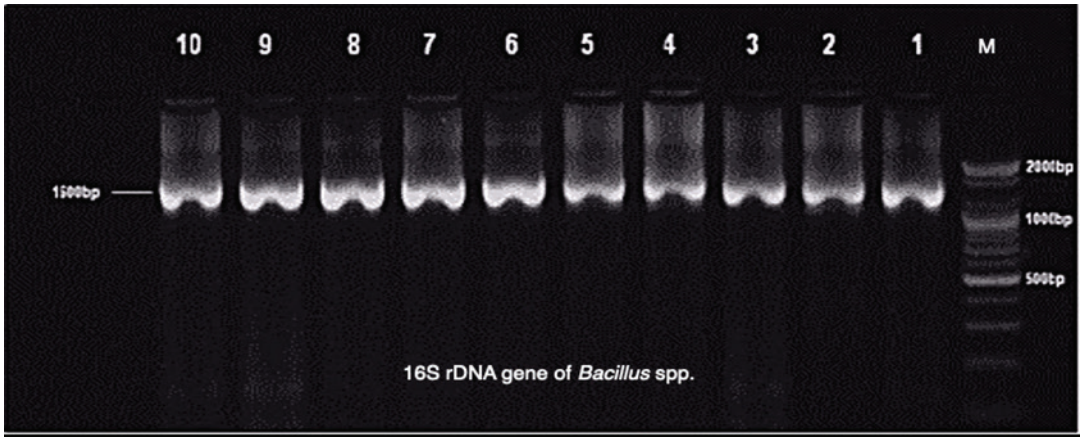

Fig. 1. Bands of agarose gel electrophoresis for 16S rDNA gene (*Bacillus* spp.); M:2,000 bp DNA Marker, the band size of 10 samples characterized by 1,500 bp.

products must be regularly monitored to ensure the safety of consumers and avoid conceivable outbreaks of milk-borne diseases. A wide range of pathogenic bacteria can survive and persist in their growth under optimum temperature and relevant pH conditions. Hence, pasteurization and good preservation regimes can extend the shelf life of milk products and constrain bacteria thrives in marketplaces.

The prevalence of contaminated milk products with different isolates was estimated as (mean: 50%, 95% Cl: 36.8–63.2). Other studies from Ethiopia and Nepal estimated the prevalence of contamination of milk products with microorganisms as 52% and 41%, respectively (Berhe *et al*., 2020; Rizal *et al*., 2023). Different risk factors are associated with contamination of milk products with pathogenic bacteria, including unhygienic milking practices, poor hygienic experiences, unsanitary utensils, and insufficient pasteurization of milk (Kouamé-Sina *et al*., 2012). In addition, a high rate of isolated bacteria could be linked with antibiotics-resistance, especially those that produce Extended-spectrum β-lactamases by *E. coli* and *K. pneumoniae* (Shaikh *et al*., 2015; Asfaw *et al*., 2023). Hence, implementing proper cleaning and sanitation can significantly lower the probability presence of pathogenic microbes in dairy products.

Table 3. Identified bacterial strains using the sequences of 16S rDNA gene.

Bacillus species	Strain/Substrain	% identical
Bacillus amyloliquefaciens	Subsp. strain Nk8-22	99
Bacillus amyloliquefaciens	Strain ORE3	100
Bacillus subtilis	Strain H171	100
Bacillus subtilis	Strain b18	100
Bacillus subtilis	Subsp. subtilis str. 168	100
Bacillus subtilis	Strain CO-6	100
Bacillus subtilis	Strain H210315C21	100
Bacillus subtilis	Subsp. spizizenii	96
Bacillus cereus	Strain FDAARGOS_797	100
Bacillus cereus	Strain NRC215	100

There is a significant difference between the means of TBC of samples collected, and the greatest mean was found in cheese samples 7.29 ± 2.70 log 10 CFU/ml (Table 1). Similar to our results were reported from Ethiopia by Aliyo and Teklemariam (2022), with the average total bacteria TBC estimated as 7.57 ± 0.83 log CFL/ml in raw milk. Another study confirmed the total mean count of microbial assessment was estimated between 2.73 and 7.30 from cheese and milk samples gathered from various marketplaces in Tanta city of Egypt (Heikal *et al*., 2014). The contamination of milk products with a high average of TBC may be due to cheese produced in the absence of hygienic measures, unclean equipment used, and imperfect cooling store, resulting significant multiply of bacteria. The hazard analysis critical control point must be applied at the retail marketplaces to reduce the likelihood of infection by food-borne diseases (Bacigale *et al*., 2023).

In this research, different genus and strains of pathogenic bacteria were detected, notably *S. aureus*, *E. coli*, and *P. aerogenosa* (Table 2). A study survey was undertaken by Khalid and Abbas (2021) involving 150 samples from which the bacteria, *Yersinia enterocolitica* was detected in 8% of bovine and ovine milk samples collected from local markets of Basrah. A range of bacteria species of *Enterobacteriaceae* were also reported from different dairy products sold in Baghdad and Al-Muthanna marketplaces in Iraq (Al-Rudha *et al*., 2021; Azeez and Makki, 2023). The contamination of dairy products by certain types of microorganisms is obviously related to the preharvest and post-harvest process of milk production and maybe due to bad storage conditions. A group of lactoperoxidase enzymes (LPO) including LPO, thiocyanate, and hydrogen peroxide exist in natural milk that is more useful as preservatives for improving the shelf life of milk and inhibiting microbe growth in milk (Wolfson and Sumner, 1993; Al-Baarri *et al*., 2019). A key component in the prevention of bacteria growth is to increase CO_2 concertation and lower the temperature of milk products, where they are stored (Martin *et al*., 2003).

According to the findings of our investigation, a total of 10 *Bacillus* strains were identified from isolates. *Bacillus amyloliquefaciens* and *B. subtilis* are identified strains in the present study through sequenced 16S rDNA gene (Table 3). Contaminated dairy products by these strains do not constitute any concern to human health and have been used as probiotics to increase milk output or biocontrol in agriculture implications (Sun *et al*., 2013; Zalila-Kolsi *et al*., 2023). One experiment with 28 Holstein-Friesian cows in Thailand when fed them *B. subtilis* at a dose of 2×10^{11} CFU/day, resulted in an increase in milk outputs by 1.7 kg per day (Choonkham *et al*., 2020). *Bacillus cereus* is an important foodborne pathogen also detected in the dairy cheese samples, and this indicates the contamination may be sourced either from the farm environment or septic of milk plants. The highest ASF count in the study carried out in India was determined to be 108 CFU/ml, with the prevalence of *B. cereus* in milk products ranging from 33% to 55% (Kumari and Sarkar, 2014). The spore of *B. cereus* can tolerate pasteurization temperature (Ibrahim *et al*., 2022); therefore, it can be controlled by using natural antibacterial agents, and irradiation, in addition to good hygiene management at the farm and milk plants (Yang *et al*., 2023).

Conclusion

This study was carried out to assess the microbiological quality of dairy products. According to study findings, the surveyed milk products were highly contaminated with different harmful bacteria. Aerobic and spore-forming bacteria counts seem to be significantly higher in homemade cheese and raw milk samples. Further studies are needed not only to investigate other biological hazards in dairy products but also to evaluate sanitation conditions of marketplaces and plant milk, as well as the sources of products where natural milk is derived. Adoption of food safety management is a good strategic intervention that provides food safety for consumers and prevents the spread of food-borne diseases or outbreaks in human populations.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Authors contributions

All authors contributed to this study. All authors read and approved the final manuscript.

Data availability

All data are provided in the manuscript.

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