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Antimicrobial susceptibility patterns of infective *Streptococci* recovered from local and imported mozzarella cheese from different markets in Baghdad

Moutaz A.W. Abdul Mounam¹ (D), Basil R.F. Razook² (D) and Entesar Hussain Madi^{2*} (D)

¹Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq ²Department of Zoonoses Research Unit, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

ABSTRACT

Background: This research identified *Streptococci spp.* depending on culture, biochemistry, the Visual Information Technologies (VITEK) technique, ability to produce biofilms, and antibiotic resistance.

Aim: The goal of this study was to perform microbiological procedures to evaluate the qualitative qualities of mozzarella cheese against infective *Streptococci* using microbiological care.

Methods: Sixty (60) mozzarella cheese samples were brought from diverse markets in Baghdad from October 2023 to December 2023 at the Zoonoses Research Unit and Veterinary Public Health Department, Veterinary Medicine College, University of Baghdad. Culture of samples on agar (MacConkey and blood) and aerobically incubated at 37°C for 48 hours. Gram staining purified colonies to notice Gram-positive cocci arranged in strings that were supposed to be *Streptococcus species*, and then sub cultured on nutrient agar for identification using the VITEK technique. The isolates were tested for their capacity to lyse human red blood cells by streaking blood agar and incubating at 37°C for 24 hours. *Streptococcal* isolates were streaked on a Congo-red medium. All isolates were inspected for antibiotic susceptibility using 15 common antibiotics via the disc diffusion method.

Results: Isolates mozzarella cheese samples 8 (13.3%), these (eight) isolates include: (*Streptococcus thoraltensis, Streptococcus sanguinis, Streptococcus cremoris,* and *Streptococcus alactolyticus*. The susceptibility of *S. thoraltensis, S. sanguinis,* and *S. alactolyticus* was (26.6%), and *S. cremoris* (20%) to all antibiotics. Resistance of *S. thoraltensis was* (53%), *S. sanguinis, S. alactolyticus* were (66%), and *S. cremoris* was (73%) to all antibiotics. *S. thoraltensis, S. cremoris,* and *S. alactolyticus* were susceptible (75%) to Amikacin. *S.sanguinis* and *S. cremoris* were susceptible (50%) to Tigecycline. *Streptococcus thoraltensis* and *S. alactolyticus* were susceptible (50%) to Ciprofloxacin. *Streptococcus thoraltensis* and *S. sanguinis* were susceptible (50%) to Azithromycin. *Streptococcus sanguinis* was susceptible (25%) to vancomycin. *Streptococcus alactolyticus* was susceptible (25%) to Streptococcus thoraltensis was susceptible (25%) to Penicillin. All isolates were 100% resistant to imipenem, lincomycin, meropenem, methicillin, and chloramphenicol.

Conclusion: Analysis of mozzarella cheese samples identified four predominant *Streptococcus species* and their antibiotic activity.

Keywords: Streptococci isolates, Streptococcus spp., Antibiotics susceptible, Mozzarella cheese, Unpasteurized milk.

Introduction

The genus *Streptococcus* contains both commensal and harmful species. It has experienced tremendous extension and revision as a result of the speedy development of technologies for molecular identification and microbial phenotyping (Lu *et al.*, 2016; Tian *et al* 2019). *Streptococcus* has about 100 recognized *species*, numerous of which are nurses or symbiodinium in people and animals (Foster *et al.*, 2020). Antibiotic resistance is transformed from beneficial and commensal bacteria to pathogenic bacteria through the docximportant role of the food chain (Flórez and Mayo, 2017). *Streptococci* are widely spread in humans and animals, and they can cause various diseases (Dhanda *et al.*, 2013). They are present in the oral and intestinal regions of animals and humans, as well as in raw milk, dairy products, and plant material (Tsakalidou *et al.*, 1998). The source of the milk (sheep or cows), method of production (unpasteurized milk and pasteurized or aging period), production, conditions, and other factors affect the microorganisms in the cheese (Verraes *et al.*, 2014; Yao *et al.*, 2022).

*Corresponding Author: Entesar Hussain Madi. Department of Zoonoses Research Unit, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq. Email: *Intisar.h@covm.uobaghdad.edu.iq*

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Dairy products, due to their short shelf life, serve as an ideal growth medium for a diverse array of microorganisms (Ledenbach and Marshall, 2009; Garnier et al., 2017; Rauh and Xiao, 2022). Therefore, it is important to monitor the microbiological quality of dairy (products), especially the whole viable count and concentration of *Escherichia coli* bacteria, as they are indicators of the sanitary condition of these (products) (Ryu et al., 2012; Pal et al., 2016). The concentration of lactic acid bacteria (LAB) in dairy products such as mozzarella cheese, which are the main components of the starter culture, should be monitored (Mazzoli et al., 2014; Blaya et al., 2018). Fermented products enhance the flavor and consistency of cheese while preventing food spoilage bacteria by producing substances that hinder their growth (Najim, 2010; Algboory et al., 2017; Hussein and Jabbar, 2020). Cheese makers must frequently observe the concentrations of LAB and bacteria during the production of fresh cheese to ensure the quality and safety of their products (Losito et al., 2014). Antimicrobial resistance is a global problem (Watkins and Bonomo, 2016; Motaweg and Naher, 2017; Zhang et al., 2019).

Antibiotic-resistant bacteria have been identified in various foods (Wang *et al.*, 2012; De Jong *et al.*, 2013). Devirgiliis *et al.* (2013), Gad *et al.* (2014), and Soares-Santos *et al.* (2015) detailed the factors that influence antibiotic resistance in bacteria associated with dairy products. They also highlighted the widespread distribution of antibiotic resistance genes among food bacteria, emphasizing their role as reservoirs of resistance genes (Marshall and Levy, 2011; Hu *et al.*, 2013; Flayyih *et al.*, 2016; Albanna and Al-Layla, 2020; Taher *et al.*, 2020).

In a study, infection with *Streptococcus* isolated from soft mozzarella cheese, and the importance of stopping the spreading of the epidemic by using molecular tools, first by detecting pathogenic microbes in food, which pose a health hazard to consumers (Hussaini *et al.*, 2014; Mounam *et al.*, 2023).

Materials and Methods

The research was conducted from October 2023 to December 2023 at the Zoonotic Disease Unit and the Public Health Department of the Veterinary Medicine College, University of Baghdad. Sixty (60) mozzarella cheese samples were emulsified with 2% buffered sodium citrate in a stomacher for 3 minutes and subsequently cultured on (MacConkey agar and blood agar). The cultures were then incubated aerobically at 37°C for 48 hours as described by Coque *et al.* (1995). The purified colonies were subjected to Gram staining, which revealed cocci arranged in series, suggesting *Streptococcus species*. These colonies were further subcultured on nutrient agar and were identified using the VITEK method.

VITEK method

The VITEK technique was performed following the manufacturer's instructions. The isolated colonies were moved into a polystyrene tube filled with a saline solution (NaCl) adjusted to a density range of 0.5–0.63. The tube and card were placed in the VITEK 2 cassette using the VITEK 2 Densi Check spectrophotometer. The cassette was then autoincubated inside the VITEK two instrument, and results were interpreted after 18–24 hours. This procedure was performed at the Zoonoses Unit laboratories of the Veterinary Medicine College, University of Baghdad.

Hemolysin activity

Hemolysis is a critical virulence factor in *Streptococcus* infections, influencing infection severity. *Streptococcal* isolates were evaluated for their capacity to lyse red blood cells, streak the colonies on blood agar, and incubate them at 37°C for 24 hours. The appearance of a clear zone (halo) around the colonies indicated hemolysin activity, and the type of hemolysis was recorded (Coque *et al.*, 1995).

Biofilm formation

Biofilm formation was evaluated by streaking *Streptococcal* isolates on Congo-red medium and incubating them at 37°C for 24 hours (Fig. 1). Following the method outlined by Freeman *et al.* (1989).

Antimicrobial susceptibility test

The Kirby–Bauer method uses paper disks infused with antibiotics placed on the surface of Muller–Hinton agar to assess antibiotic susceptibility. After incubating 4–5 colonies in Tryptone soy broth at 37°C until reaching a standard opacity (equal to a 0.5 McFarland standard), a sterile swab was used to spread the bacteria onto Muller–Hinton agar. After drying for 10 minutes, antibiotic disks were placed on agar with adequate spacing to prevent the overlap of inhibition zones. The diameters of these zones are measured using a vernier caliper and compared with standardized tables provided by antibiotic disk suppliers to determine minimal inhibitory concentrations (CLSI, 2022) (Fig. 1).

Results

Consistent with culture, microscopy, and the biochemical and VITEK results (Fig. 2), there were (eight) positive counts of Streptococcal isolates obtained from (60) samples from different markets. These (eight) isolates include Streptococcus thoraltensis, Streptococcus sanguinis, Lactococcus lactis subspecies cremoris, and Streptococcus alactolyticus which include (two) isolates of each. In this research, there was a frequency distribution of isolated microorganisms from examined cheese 8 (13.3%) (Table 1). Black, dry crystalline colonies signified strong biofilm formation, while dark colonies without dry crystalline structures suggested intermediate biofilm formation. The pale pink colonies indicate the absence of biofilm formation (Fig. 1). The detailed antibiotic activity



Fig. 1. (A) Antimicrobial susceptibility of *Streptococcus isolates* on modified Muller-Hinton agar. (B) *Streptococcal* isolates were cultured in Congo red medium.

results provide a comprehensive overview of the susceptibility patterns of the four Streptococcus species to the tested antibiotics. The susceptibility of S. thoraltensis, S. sanguinis, and S. alactolyticus were (26.6%) and Streptococcus cremoris (20%) to all antibiotics. Resistance of S. thoraltensis was (53%), S. sanguinis, S. alactolyticus were (66%) and S. cremoris was (73%) to all antibiotics (Table 3). Streptococcus thoraltensis, S. cremoris, and S. alactolyticus were susceptible (75%) to Amikacin. S. sanguinis and S. cremoris were susceptible (50%) to Tigecycline, S. thoraltensis, and S. alactolyticus were susceptible (50%) to Doxycycline. Streptococcus sanguinis and S. alactolyticus were susceptible (50%) to Ciprofloxacin. S. thoraltensis and S. sanguinis were susceptible (50%) to Azithromycin. S. sanguinis was susceptible (25%) to vancomycin. Streptococcus alactolyticus was susceptible (25%) to Streptomycin. S. thoraltensis was susceptible (25%) to Amoxicillin and Clavulanic acid. S. cremoris was susceptible (25%) to Penicillin. All isolates were 100% resistant to imipenem, lincomycin, meropenem, methicillin, and chloramphenicol.

It is evident that certain antibiotics, such as Tigecycline and Amikacin, show varying degrees of effectiveness against different species, whereas others, like Imipenem and Lincomycin, exhibit consistent resistance across all strains (Tables 2, 4, and 5).

Discussion

Mozzarella cheese is made specifically from buffalo milk, in addition to other types of animal milk (Araujo *et al.*, 2012). According to Ammin *et al.* (2024) study, *Streptococcus agalactiae* and *Streptococcus pyogenes* were identified as the primary *Streptococcus species* responsible for pharyngitis in children, with prevalence rates of 24% and 18%, respectively, the research

also highlighted the variation in bacterial species distribution based on the patients' ages. Furthermore, antibiotic sensitivity testing revealed intriguing results; Streptococcus pneumoniae and Streptococcus parasanguinis exhibited high sensitivity to multiple antibiotics, whereas S. pyogenes demonstrated resistance to Metronidazole and Azithromycin (Ammin et al., 2024). Vásquez-García et al. (2017) found isolation from milk buffalo were 11(5.5%). Morea et al. (1999) and Al-Khafaji et al. (2013) examined cheese to investigate the dominant bacteria for the detection of potential pathogens in raw milk-derived unripe cheese. They identified Streptococcus uberis along with other species such as Corynebacterium, Streptococcus, Enterococcus, Staphylococcus, Leuconostoc, and Lactococcus.

In Baghdad–Iraq, the first case of vancomycin resistance in raw milk was reported by Al Marjani *et al.* (2016). The results of sensitivity to vancomycin (25%) were accepted by o Vásquez-García *et al.* (2017) and noncompatible (12.5%) for penicillin. Bhardwaj *et al.* (2018) found in India beta-hemolytic *Streptococci* less resistant to ciprofloxacin (21, 9.5%). On the other hand, all isolates of *Enterococcus faecalis* in crude milk and imported milk powders at Baghdad markets are resistant to vancomycin (Al-Shammary, 2019). Antimicrobial resistance in *Streptococci* from animals can differ significantly based on the specific *Streptococcal* type, so it should not be considered a single entity (Haenni *et al.*, 2018).

Antibiotic treatment plays a critical role in managing mastitis infections. Understanding the prevalence of antibiotic resistance and the susceptibility of microorganisms isolated from infected cow milk could improve mastitis treatment strategies. Thus, infection treatment with intramammary antibiotics begins before -

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Fig. 2. VITEK results of Streptococcal isolates.

Table 1. Distribution of isolated from check cheese (60 samples).

| Isolated organism | No. of isolates | % |
|-------------------|-----------------|------|
| 8 | 60 | 13.3 |

| Antibiotic | S.1 | S.2 | S.3 | S.4 |
|----------------------------|-----|-----|-----|-----|
| Nitrofurantoin | İ | Ŕ | Ŕ | Ŕ |
| Tigecycline | İ | Ś | Ś | İ |
| Imipenem | Ŕ | Ŕ | Ŕ | Ŕ |
| Amikacin | Ś | Ŕ | Ś | Ś |
| Lincomycin | Ŕ | Ŕ | Ŕ | Ŕ |
| Vancomycin | Ŕ | Ś | Ŕ | Ŕ |
| Meropenem | Ŕ | Ŕ | Ŕ | Ŕ |
| Streptomycin | Ŕ | Ŕ | Ŕ | Ś |
| Doxycycline | Ś | İ | Ŕ | Ś |
| Ciprofloxacin | İ | Ś | İ | Ś |
| Amoxicillin Calvuanic acid | Ś | Ŕ | Ŕ | Ŕ |
| Methicillin | Ŕ | Ŕ | Ŕ | Ŕ |
| Azithromycin | Ś | Ś | Ŕ | Ŕ |
| Penicillin | Ŕ | Ŕ | Ś | Ŕ |
| Chloramphenicol | Ŕ | Ŕ | Ŕ | Ŕ |

Table 2. Average diameters of antibiotic inhibition zones (mm).

S.1 = Streptococcus thoraltensis; S.2 = Streptococcus sanguinis; S.3 = Streptococcus Cremoris; S.4 = Streptococcus alactolyticus.

 Table 3. Antibiotic susceptibility of isolates.

| Isolates | Resistance | Intermediate | Sensitive |
|-----------------------------|------------|--------------|-----------|
| Streptococcus thoraltensis | 8 (53.3%) | 3 (20%) | 4 (26.6%) |
| Streptococcus sanguinis | 10 (66.6%) | 1 (6.66%) | 4 (26.6%) |
| Streptococcus cremoris | 11 (73%) | 1(6.66%) | 3 (20%) |
| Streptococcus alactolyticus | 10 (66.6%) | 1(6.66%) | 4 (26.6%) |

Table 4. Percentage of antibiotic sensitivity to Streptococcus species.

| Antibiotic | (Ś %) |
|----------------------------|--------------------------------|
| Nitrofurantoin | 0 percent (S.1, S.2, S.3, S.4) |
| Tigecycline | 50 percent (S.2, S.3) |
| Imipenem | 0 percent (S.1, S.2, S.3, S.4) |
| Amikacin | 75 percent (S.1, S.3.S.4) |
| Lincomycin | 0 percent (S.1, S.2, S.3, S.4) |
| Vancomycin | 25 percent (S.2) |
| Meropenem | 0 percent (S.1, S.2, S.3, S.4) |
| Streptomycin | 25 percent (S.4) |
| Doxycycline | 50 percent (S.1, S.4) |
| Ciprofloxacin | 50 percent (S.2, S.4) |
| Amoxicillin Calvuanic acid | 25 percent (S.1) |
| Methicillin | 0 percent (S.1, S.2, S.3, S.4) |
| Azithromycin | 50 percent (S.1, S.2) |
| Penicillin | 25 percent (S.3) |
| Chloramphenicol | 0 percent (S.1, S.2, S.3, S.4) |

S.1 = Streptococcus thoraltensis; S.2 = Streptococcus sanguinis; S.3 = Streptococcus Cremoris;

S.4 = *Streptococcus alactolyticus.*

Table 5. Proportion of antibiotics resistant to *streptococcus* species.

| Antibiotic | Ŕ % |
|-----------------------------|----------------------------------|
| Nitrofurantoin | 75 percent (S.2, S.3, S.4) |
| Tigecycline | 0 percent (S.1, S.2, S.3, S.4) |
| Imipenem | 100 percent (S.1, S.2, S.3, S.4) |
| Amikacin | 25 percent (S.2) |
| Lincomycin | 100 percent (S.1, S.2, S.3, S.4) |
| Vancomycin | 75 percent (S.1, S.3, S.4) |
| Meropenem | 100 percent (S.1, S.2, S.3, S.4) |
| Streptomycin | 75 percent (S.1, S.2, S.3) |
| Doxycycline | 25 percent (S.3) |
| Ciprofloxacin | 0 percent (S.1, S.2, S.3, S.4) |
| Amoxicillin Clavulanic acid | 75 percent (S.2, S.3, S.4) |
| Methicillin | 100 percent (S.1, S.2, S.3, S.4) |
| Azithromycin | 50 percent (S.3, S.4) |
| Penicillin | 75 percent (S.1, S.2, S.4) |
| Chloramphenicol | 100 percent (S.1, S.2, S.3, S.4) |

S.1 = Streptococcus thoraltensis; S.2 = Streptococcus sanguinis; S.3 = Streptococcus Cremoris; S.4 = Streptococcus thoraltensis; S.2 = Streptococcus sanguinis; S.3 = Streptococcus cremoris; S.4 = Streptococcus sanguinis; S.3 = Streptococcus cremoris; S.4 = Streptococcus sanguinis; S.3 = Streptococcus cremoris; S.4 = Streptococcus sanguinis; S.3 = Streptococcus cremoris; S.4 = Streptococcus sanguini

S.4 = *Streptococcus alactolyticus*.

microbiological culture (Wongkattiya, 2008; Kanaan and AL-Shammary, 2013; Muruzović *et al.*, 2018).

Antibiotic resistance jeopardizes the effective prevention of infectious diseases. While some bacterial strains are naturally resistant, others develop resistance through mutation, recombination of foreign DNA into their chromosome, or horizontal gene transfer (Brown-Jaque *et al.*, 2015; Petrovich *et al.*, 2020; Zarei-Baygi and Smith, 2021).

Conclusion

The primary objective of this study was to conduct a comprehensive microbiological assessment of mozzarella cheese samples, with a specific focus on detecting and evaluating the presence of infective Streptococci. This goal was driven by the need to ensure the highest quality and safety standards in the cheese industry, as well as to address potential health concerns associated with Streptococcal infections. By employing a range of microbiological procedures, we aimed to gain insights into the qualitative aspects of mozzarella cheese, particularly its susceptibility to Streptococcal contamination. The ultimate goal was twofold: first, to establish a robust framework for identifying and characterizing Streptococcal strains in cheese samples, and second, to develop effective strategies for microbiological care and control. The significance of this study lies in its potential to enhance food safety measures and protect public health. Streptococci, particularly certain pathogenic strains, pose serious health risks when present in food products. By understanding the prevalence and behavior of these

bacteria in mozzarella cheese, researchers can develop targeted interventions and guidelines to minimize the risk of Streptococcal infections. The findings of this study can contribute to the development of improved cheese manufacturing processes, storage conditions, and sanitation practices. By identifying specific Streptococcal species and their antibiotic susceptibility profiles, the cheese industry can implement tailored measures to prevent contamination and ensure the production of safe and high-quality mozzarella cheese. In conclusion, the ultimate goal of performing microbiological procedures to evaluate mozzarella cheese against infective Streptococci is to safeguard public health, improve food safety standards, and enhance the overall quality of this popular dairy product. The insights gained from this study will undoubtedly benefit both consumers and the cheese industry by fostering a culture of microbiological care and excellence.

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The authors declare no conflict of interest.

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The authors declare that finance is personal.

Authors' contributions

The authors declare that contributions to the research are equal.

Data availability

All data supporting the research is available within the manuscript.

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