Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 30/07/2023;
Date Revised: 31/08/2023;
Date Published Online: 30/09/2023;
Date Updated: 06/09/2025

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How to Cite:
Mounam MAWA, Al-Gburi NM, Razook BRF (2023). Investigation of pathogenic *Streptococci* species in local and imported mozzarella soft cheese in Baghdad city. Adv. Life Sci. 10(3): 486-490.

Keywords:
Streptococcus species; Gene sequencing; Mozzarella cheese

Editorial Note:
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Molecular Identification of *Streptococcus* and *Lactococcus* Species in Local and Imported Mozzarella Cheese in Baghdad City

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Abstract

Background: The present work investigated the diversity of Streptococcus and related species isolated from local and imported mozzarella soft cheese in Baghdad City from October 2022 to January 2023. The study aimed to examine the molecular characterization of 16Sr RNA gene in some streptococcus species isolates from mozzarella soft cheese in Baghdad city

Methods: From 50 samples, 8 isolates in all were found and identified based on the VITEK, molecular, and sequencing of the 16SrRNA gene. The eight isolates represented *Lactococcus cremoris. Streptococcus alactolyticus, Streptococcus sanguinis, and Streptococcus thoraltensis.* The isolates were subjected to conventional PCR and electrophoresis to detect the 16SrRNA gene using specific primers , Streptococcal genes were used in the neighbor-joining phylogenetic analysis that determined these sequences were derived from Streptococcal genes.

Results: DNA sequencing evaluation Significant alignments (96-99% identities) to the *Streptococci* isolates found in BLAST-NCBI Gene-bank were discovered after sequencing the bacterial DNA products generated by PCR. The results revealed that 8/50 amplified the 16SrRNA gene, which has a molecular weight of about 1250 bp. the gene distribution was statistically significant ($p \leqslant 0.05$).

Conclusion: This study successfully identified several species of *Streptococcus* and *Lactococcus* from mozzarella cheese samples using molecular methods. The findings reveal a mix of expected dairy-associated bacteria (*L. cremoris*) and species that can be opportunistic pathogens (*S. sanguinis, S. alactolyticus*). While not all identified isolates are pathogenic, the presence of opportunistic species highlights the need for consistent quality control and hygiene in cheese production to minimize any potential risk to consumers.

Introduction

Streptococci are stained positive with gram stain, also they are non-motile and non-spore forming bacteria, responsible for fermentative spoilage of dairy products. *Streptococci* are catalase-negative, facultative anaerobes that are arranged in pairs or chains, most Streptococci have these features and some of them are obligate aerobes [1]. *Streptococci* are categorized based on colony morphology, hemolysis, biochemical reactions, and—most definitively—serologic specificity. They are divided into three groups according to the type of hemolysis that was visible on blood agar: non-hemolytic (no hemolysis), alpha-hemolytic (incomplete, green hemolysis), and beta-hemolytic (clear, complete lysis of red cells). The basis for serologic classification is the antigenic diversity of the polysaccharide capsule, cell wall pili-associated protein, and cell wall carbohydrates of Group B Streptococci [2].

New species are continuously being discovered in samples from people, animals, and the environment. They have been identified using phenotypic criteria and Comparisons of 16SrRNA gene sequences [3]. The hemolytic reaction, group carbohydrate antigens (Lancefield serotyping), and phenotypic assays were initially used to identify streptococci. The identification of species of Streptococcus has been proposed using a variety of molecular DNA-based techniques.

There have been several new additions to the *viridans streptococci* family over the previous ten years, from which these species or subspecies identified in our study that belong to *Streptococci as thoraltensis (Genus sensu stricto), cremoris, alactolyticus and sanguins spp.* [4].

It is well known that the microbial community in raw milk is complex. These microorganisms include technologically significant bacteria like Lactic Acid Bacteria (LAB), which can support favorable fermentative outcomes processes. Culture-based methods are used to traditionally identify the type of bacteria found in milk [5].

A lactic acid bacteria called *Lactococcus lactis* is frequently found in milk and fermented dairy products for particular types of cheese*, L. lactis subsp. cremoris* strain is the recommended starter. They are well-known for their distinctive aroma profiles and their enhanced milk growth response. Controversial cremoris strains have been found in milk and naturally fermented foods [6].

Uncertainty surrounds *S. thoraltensis* clinical relevance as a human pathogen. [7]. first identified this peculiar microbe after isolating it from swine intestinal tracts. We know very little about this strain's potential for infecting humans, and to our knowledge, no cases of this organism's infection of humans have ever been documented. According to the literature, this unusual and uncommon species of streptococci was only recently identified from human samples. *S. thoraltensis* was shown to be the main colonizing isolate in the oropharynx and nasal cavities of 29 fuel workers, according to a recent study [8].

Traditional mozzarella is a soft cheese with a high moisture content (50–60%) that is frequently dipped into a cold governing liquid. [9]. Under these circumstances, mozzarella is preserved at 4 °C for 10 to 12 days while being stored, keeping its soft, springy texture and high levels of expressible serum. Fresh cheese like mozzarella has undergone numerous attempts to manage its rotting microorganisms. Carbon dioxide-based modified atmospheres effectively inhibited psychrotrophs and inhibited staphylococci, molds, and yeasts while stabilizing lactic and mesophilic flora [10].

Methods

Ethical agreement

The Veterinary Public Health Laboratories and the Zoonotic Diseases Unit both gave their approval for this study. College of Veterinary Medicine at the University of Baghdad.

A total of (50) samples of local and imported shredded mozzarella soft cheese (fat in dry matter 40% and moisture max. 50%). Used for pizza were randomly collected from different markets in Baghdad city from October 2022 till January 2023. All samples were delivered to the lab in the Department of public health university of Baghdad by ice box and kept in refrigeration under 4 °C until analysis.

Bacterial identification

10 g of cheese in sterile collecting bags was homogenized with 90 ml of 2% (w/v) sodium citrate solution using a stomacher for five minutes. Samples were serially diluted (tenfold dilution) in the physiological solution and then streaked on the blood agar and incubated aerobically for 24 hours at 35-37 °C. The purified small, pinpoint white-greyish, hemolytic or not hemolytic colonies were stained with Gram stain to detect Gram-positive cocci arrangement as chains that suspected Streptococcus spp. and then subculturing on nutrient agar to identified by VITEK technique [11].

VITEK technique was done according to manufacturer instructions. Pure colonies were transferred into a polystyrene tube containing saline NaCl for a density equivalent to (0.5-0.63) utilizing the VITEK 2 Densi Check spectrophotometer, then the tube and card were put into the VITEK 2 cassette and the card was auto inoculated inside the VITEK 2

instrument, the result was read after (18-24) hour. VITEK was done in laboratories of the zoonotic disease unit at the University of Baghdad.

Molecular Detection

Using the Gene Aid Genomic DNATM Kit, the genomic deoxyribonucleic acid (DNA) was extracted following the manufacturer's instructions(GeneAid Research, USA). To conduct molecular analyses using Polymerase Chain Reaction (PCR), the extracted DNA samples were stored at -20°C. Using multiplex PCR, 8 isolates of streptococci were examined. In this investigation, 16SrRNA primer sets were used to identify the virulence genes, as shown in Table 1. The primers were lyophilized, then dissolved in free ddH2O to give a final concentration of 100 pmol/µl as stock solution and keep stock at - 20 °C to prepare 10 pmol/µl concentration as work primer suspension. This mixture resulted in a final volume of 100 µl with a working concentration of 10 pmol/µl.

Primers	Type	Primer Sequence	Type
16s RNA	F	5'- AGAGTTTGATCCTGGCTCAG- 3'	1250bp
	R	5'- GGTTACCTTGTTACGACTT- 3'	

Table 1: The primer sequences used in this study.

Polymerase chain reaction

The Applied BiosystemsTM ProFlexTM PCR System from Fisher Scientific/USA was used for the amplification. 35 rounds of 45-second denaturation are performed after an initial denaturation of five minutes at 95 degrees Celsius. annealing at 57 °C for 45 seconds, extension for (1 min) at 72 °C 35 cycles, and final extension for 5 minutes at 72 °C one cycle.

Gel electrophoresis

Dissolve 1.5g of agarose powder in 100 mL of 1x TBE and melted in a hot block to prepare an agarose solution. Waiting until the solution cools. 3 µl ethidium bromide was then added, A 100 bp DNA marker from New England Biolab was used as a measure of molecular size. DNA samples (5 µl) were loaded into agarose gel wells after being combined with 3µl of DNA loading buffer. At 70 V, 65 mA for an hour, the agarose gel electrophoresis was completed. The DNA was examined using a UV transilluminator.

DNA sequencing

The PCR products of the 16S rRNA gene from eight Streptococcus isolates were sent for sequencing to Macrogen (Korea), along with the corresponding forward and reverse primers. Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). MEGA6 was used to edit, align, and compare the sample sequences (MT807294.1, MT597548.1, OM658620.1, and MT550017.1) with the standard sequencing. [12]. Using the MEGA7 program, a phylogenetic tree was created for each gene sequence [13].

Results

Phenotypic description of *Streptococcus* isolates: According to cultural, microscopic, and VITEK 2 features the positive number of streptococci isolates were (8) obtained from a total of (50) samples from different markets. isolates produced hemolysis on blood agar. Hemolysis is one of the streptococci virulence factors that play an essential role in the severity of infections.

Detection of 16SrRNA gene: Each of the 8 Streptococcus isolates, isolated from mozzarella soft cheese, that was used for DNA extraction were precisely identified using the prior techniques., Agarose gel electrophoresis was used to find the results. All streptococcus isolates were subjected to molecular analysis, and the results revealed that 8/50 amplified the 16SrRNA gene, which has a molecular weight of about 1250 bp as shown in Figure 1. the gene distribution was statistically significant ($p \leqslant 0.05$). By using molecular detection from mozzarella soft cheese media of streptococcus, the current study aimed to shed light on the prevalence of the 16SrRNA gene as a virulence factor in Baghdad, Iraq.

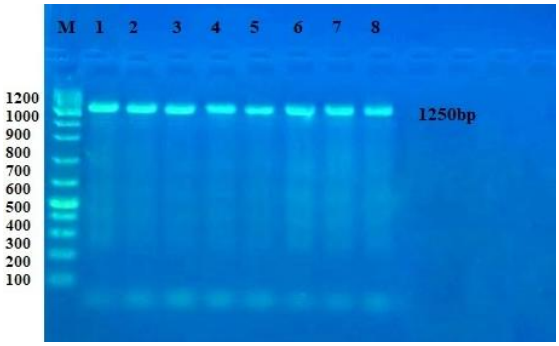

Figure 1: The band sizes of the PCR result. The process used 1.5% agarose and a 5-volt/cm2 voltage. 1:30 hours of 1x TBE buffer. M: DNA ladder (100).

Gene sequencing: The two chosen phenotypic and molecular isolates underwent 16SrRNA gene partial DNA sequencing. To determine potential genotypic differences, a UPGMA method format file comprising the local strain sequences was employed to analyze the molecular relationship between isolates from Baghdad City, Iraq, and other global sequencing submitted to Gene-bank. The streptococci species, Gene-bank's official accession numbers of (MT807294.1, MT597548.1, OM658620.1, and MT550017.1) When compared using the MEGA7 program, the four species showed a phylogeny percentage of 99%. According to Figure (2, 3, and 4). In molecular typing methods, the sequence diversity within individual genes can be

utilized to identify the relatedness of bacteria. A public database called the International Nucleotide Sequence Database Collaboration (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome), is receiving an increasing number of really complete bacterial genomes. The results of this study, which used high-throughput amplicon sequencing technology, gave information about the microbiota of mozzarella cheese prepared using conventional techniques. The current study's findings revealed eight positive isolates from a total of fifty samples taken from various markets. Isolates as *Lactococcus cremoris*. *S. alactolyticus*. S. Sanguinis and *S. thoraltensis*. The main bacterial group discovered was *S. alactolyticus*, as would be predicted for a fermenting dairy product. *Lactococcus lactis* spp. *cremoris* is regarded as mesophilic dairy starter culture, it is used in cheese production with or without *Lactococcus lactis*. *L. cremoris* does not give ammonia from arginine and it produces high levels of B-galactosidase, so it is useful for people suffering from lactose intolerance besides *L. cremoris*. Is low in lactose fermentation (weakly acidifying culture) with a comparison with *L. lactis* [21-24].

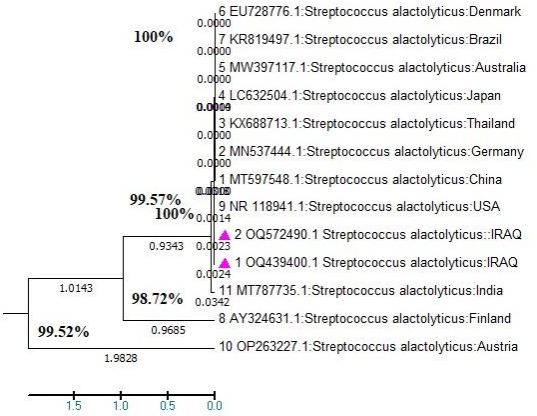

Figure 2: Comparison between Iraqi isolates and other countries of *Streptococcus alactolyticus* as determined by the UPGMA method.

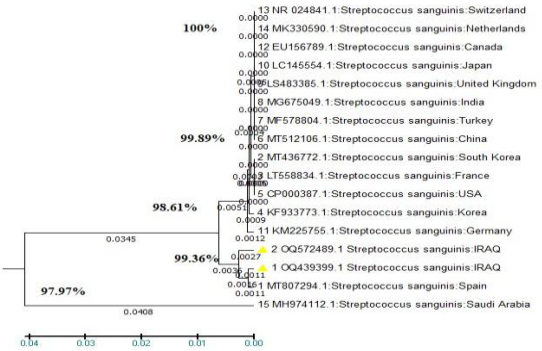

Figure 3: Comparison between Iraqi isolates and other countries of *Streptococcus sanguinis* according to the UPGMA methodology.

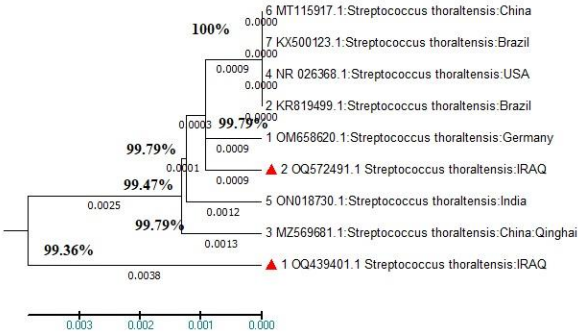

Figure 4: Comparison between Iraqi isolates and other countries of *Streptococcus thoraltensis* according to the UPGMA methodology.

Discussion

Despite the ongoing controversy regarding the function of many *streptococcus* virulence factors [14]. In molecular typing methods, the sequence diversity within individual genes can be utilized to identify the relatedness of bacteria. This technique is also beginning to have applications in the identification and classification of microbes [15]. The evolutionary analysis with the 16SrRNA gene of the four streptococci species exhibited their strong relatedness. However, the remaining strains that are similar, that isolated from France, China, Ireland, India, USA, Italy, Spain, New Zeeland, South Korea, and other countries seem to be relatively close to the local isolates, 16 srRNA gene percentage of each other countries disagreed with our results [16]. When compared with phylogenetic trees produced from previously documented sequence comparisons of various genes, the 16SrRNA gene's sequence comparison tree has the most stable nodes. Several bacteria species' differentiation has already been assessed using the 16SrRNA gene sequences. We identified the 16SrRNA gene sequences of Four frequently observed species of streptococci bacteria in soft Mozzarella cheese in the current investigation. The species identified in this study represent a mixed group in terms of their relevance to human health. The presence of *Lactococcus cremoris* is expected, as it is a common starter culture used in dairy fermentation. Conversely, the clinical significance of *Strepetococcus thoraltensis* remains uncertain. Species such as *Streptococcus sanguinis and Streptococcus alactolyticus* are known commensals but can also act as opportunistic pathogens, particularly in immunocompromised individuals. Therefore, their presence in a ready-to-eat product like mozzarella cheese underscores the importance of maintaining stringent hygienic standards during production and handling, rather than indicating a direct pathogenic threat. The sequence information showed that the 16SrRNA gene structure from *Streptococcus* spp., was comparable to that of species from other genera. There

are now more than 50 species in the genus Streptococcus, the majority of which can be placed in one of six phylogenetic clusters identified through a comparison of 16SrRNA gene sequences [17]. In the jejunal and fecal samples linked to the dogs, *Streptococcus alactolyticus* predominates among culturable lactic acid bacterium (LAB) species. An *S. alactolyticus* was found in the current study and was isolated from mozzarella cheese in Baghdad, Iraq, our results are relatively closer to results from the raw milk of [18]. *S. alactolyticus* is positive for Gram stain, cocci in shape, alpha-hemolytic, non-motile, and non-pigmented. Cheese frequently contains a type of bacteria called *Streptococcus alactolyticus*. The fermentation of cheese, a crucial phase in the cheese-making process, is aided by this bacterium because it produces lactic acid. The 16SrRNA gene sequences from type strains of different species have similarities ranging from 96% to 99%., these percentages disagreed with [19]. *Streptococcus* isolates from mozzarella soft cheese that was genetically close to one another showed many genotypic similarities. The isolates showed 99% identity to the 16S ribosomal RNA gene of *Streptococcus*. The 16S ribosomal RNA gene for strains of *Streptococcus* isolates has several substitutions, with the type of substitution (Transition, Transversion). Our result agrees with [20]. The incidence of Streptococcus isolates from mozzarella soft cheese was highlighted in the current study. In conclusion, this study highlights the utility of molecular tools for identifying the diverse bacteria present in mozzarella cheese. The detection of opportunistic pathogens like *S. sanguinis* and *S. alactolyticus*, alongside benign starter cultures, demonstrates that cheese can be a potential vehicle for medically relevant microorganisms. Therefore, our findings underscore the critical importance of strict hygiene procedures during the manufacturing process to ensure consumer safety.

Author Contributions

These authors each contributed equally.

Conflicts of interest

The authors declare no conflict of interest.

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