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Detection of Biofilm Formation and Antibiotics Resistance for Streptococcus Spp. Isolated from Some Dairy Products in Diwanyah City of Iraq

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ABSTRACT

Multidrug resistance and biofilm formation have increasingly become a leading human threat in particular dairy products, which become the primary source of essential nutrients word-wide. Biofilm formation is responsible for economic losses in the dairy industry. Therefore, in current study, the multi-drugs resistance and biofilm formation were investigated for *Streptococcus spp.* isolated different dairy products (milk, cheese, and cream) collected from various markets in Diwanyah city, Iraq. Bacteria were isolated and identified by morphological and biochemical characteristics using selective agar plates. Antibiotic resistance was tested using disc diffusion method, whereas biofilm formation was investigated using Microtiter plate method (MTP). The results showed that *Streptococcus spp.* isolates in milk (7 isolates), cheese (17isolates), and cream (18 strains) showed high resistance against novobiocin, Nalidixic acid, streptomycin, and cephalothin. However, all isolates showed high sensitivity to vancomycin. *Streptococcus* isolates showed a variant level of biofilm formation with high percentages (71.43%) of strong biofilm formation in milk isolates, 29.4% in cheese, and 50% in cream. These results suggested that multidrug resistance *Streptococcus spp*. has been observed in some dairy products with a high ability for biofilm formation and could affect the quality of dairy products and probable human threats.

KEYWORDS: Streptococcus, multidrug resistance, biofilm, dairy products, Diwanyah city.

الخلاصة

اصبحت المقاومة المتعددة للادوية وتشكيل الغشاء الحيوي تهديدًا للبشرية من خلال تلوث منتجات الالبان والتي تعتبر المصدر الرئيسي للغذاء عالميا. إلى جانب ذلك ، يعتبر تشكيل الغشاء الحيوي ذا اثر كبير للخسائر الاقتصادية في صناعة الألبان. لذلك ، تهدف الدراسة الحاليه التحري عن مقاومة بكتريا *Streptococcus spp.* للمضادات الحياتية ومقدرتها على تشكيل الغشاء الحيوي المعزولة من منتجات الالبان (الحليب والجبن والقشطة) والتي تم جمعها من أسواق مختلفة في مدينة الديوانية. تم عزل وتشخيص البكتيريا من خلال الخصائص االشكلية والتفاعلات الكيميائية الحيوية باستخدام اوساط انتقائية. تم اختبار مقاومة البكتريا المعزولة للمضادات الحيوية باستخدام طريقة انتشار القرص.أظهرت النتائج أن بكتيريا *Streptococcus*المعزولة. كانت في الحليب (7 عزلات) والجبن (17 عزلة) والقشطة (18 عزلة) والتي أظهرت مقاومة عالية ضد نوفوبيوسين وحمض الناليديكسيك والستربتومايسين والسيفالوتين. وأظهرت جميع العزلات حساسية عالية للفانكومايسين. أظهرت عزلات*Streptococcus* المستوى الشديد لتشكيل الغشاء الحيوي (71.43٪) في عزلات الحليب و 29.4٪ في الجبن ، و 50٪ في القشطة. تشير هذه النتائج إلى أن المقاومة المتعددة للادوية لبكتريا *Streptococcus* المعزولة كانت بنسب عالية في بعض منتجات الألبان ومعظم هذه العزلات ذات قدرة عالية على تشكيل الغشاء الحيوي والتي يمكن أن تؤثر على جودة منتجات الألبان وتشكل خطرا محتملا للمستهلكين.

INTRODUCTION

The incidence of multidrug resistance bacteria become raising global concerns [1]. A high impact of antimicrobial resistance bacteria was identified in different food products, particularly *Streptococcus* bacteria [2]. Taking under consideration that dairy products provide the essential nutrient that is difficult to gain from other food products [3]. Various species of Streptococcus have been identified in human and animal microflora, including *Streptococcus bovis* and *Streptococcus equinus* that include seven subspecies; these bacteria are also conceded as food fermentative and developing opportunistic pathogens [4], in addition, these bacteria are associated with an extensive range of animal and human diseases [5]. Biofilm formation is the way that bacteria occupied themselves within this enveloped structure (biofilm) and adapted their metabolism, growth rate, gene expression, and production of the protein—this ability of bacteria to adhere to the surface and occupied within polymeric extracellular cast rewrite this please [6]. Although biofilm is related to a high proportion of chronic and persistent illness by acting as a reservoir of pathogens [7], the formation of biofilm has become a significant threat of the quality of dairy products mainly that caused by *Streptococcus thermophilus*. This bacterium could persist in various parts that used throughout milk production, in which the bacterial surface proteins play an essential role in enhancing this attachment [8]. In the dairy industry, the bacterial biofilms are supposed the main contamination source of variety of dairy products and controlling the biofilm becomes crucially important [9]. These bacteria could be attached to various materials used during milk processing and forming a biofilm [10-12]. Therefore, the acidifying bacterial strain safety selection is crucial for human health and dairy industry re write try to make clear [13].

The multidrug resistance bacteria have become a problematic concern clinically and socioeconomically. This resistance could be acquired or natural [14]. Bacteria can develop resistance against the antibacterial agents, and these resistances could overcome the therapeutic effect against modern generations of antibiotics and increase, consequently, the danger of bacterial infections to humans and animals [15, 16].

MATERIALS AND METHODS

80 samples of milk (17), local cheese (33), and cream (30) dairy products were collected from various dairy shops, and supermarkets in Diwanyah city, Iraq. These samples were transferred to the laboratory without delay to be examined.

Tenfold serial dilution was applied to dilute the collected samples starting by homogenizing a (1) Gram or (1) ml of each sample in 9 ml peptone water to obtain a stock dilution. Then bacteria were isolated by spreading (100µl) from each dilution in Petri dishes containing Brain-Heart Infusion agar (BHIA) (Oxoid, UK). All plates were incubated aerobically and at 37 °C for 1-5 days. After incubation, the number of viable colonies (CFU/ml) was counted using a total viable plate count method. The selected isolates were purified by sub-culturing on the same media used for isolation. Each colony with different morphology on BHIA was isolated, subcultured, and identified [17].

Identification of Bacteria by Conventional Methods

Preliminary tests were done to identify the isolates according to the morphological and cultural characteristics. For further identification, biochemical tests were used. As a result, 32 isolates were identified. Cultural characteristics of isolated bacteria such as size, shape, pigmentation, elevation, and margin of the colony were recorded. The colonies were observed under transmitted and reflected light conditions to understand their optical properties [17]. For the identification of the isolates, selective media (MacConkey Agar, Bile Esculin Agar, Enterococcsel Agar, Eosin Methylene Blue Agar, and MRS agar). The preparations and compositions of all media used in this study were mentioned. The biochemical properties of the isolates were tested according to Bergey's Manual of Systematic Bacteriology [18]. The following tests were performed: Catalase test, oxidase test, motility test, and acid production from carbohydrates, TSI test (triple sugar-iron agar), IMVC test (indole methyl red Voges-Proskauer citrate) and Simmon citrate test. For further identification of Enterococcus sp., API 20 Strep (bioMerieux, France) was used.

Gram stain procedure was used to detect the shape, grouping, and the Gram reaction of the bacteria.

Antibiotics susceptibility assay

Antibiotic resistance of the potent biofilm-forming isolates (Milk n=7, cheese n= 17, and cream n= 18) was determined on Muller & Hinton Agar (Oxoid) using Kirby-Bauer disk diffusion method [19].

The selected antibiotics were kanamycin, K, (30µg), vancomycin, VA, (30µg), amoxicillin, AML, (10µg), erythromycin, E, (30µg), ampicillin, AMP, (10µg), cephalothin, KF,

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(30µg), chloramphenicol, C, (30µg), tetracycline, TET, (30µg), ciprofloxacin, CIP, (10µg), nalidixic acid, NA, (30µg), gentamicin CN (10µg), novobiocin, NV, (30µg), carbenicillin, CAR, (100µg), oxacillin OX (5µg), penicillin, P, (10 UI) and streptomycin, S, (10µg) were used. All the antibiotic discs were procured from Oxoid (UK). E. faecalis ATCC 33186 was used as control strains. Multiple Antibiotic Resistance (MAR Index) Index of the samples was calculated by the formula mentioned by [20].

MAR = Total number of resistance scored/ (number of isolates* Total number of antibiotics tested), [20].

Microtiter plate method (MTP)
This technique of quantitative detection of biofilm on abiotic surfaces was used [21]. After overnight incubation in brain heart infusion broth (BHIB) containing 1% glucose at 37°C, 200µL of the suspension was diluted to 1:40 and subsequently introduced into triplicates into sterile 96-well polystyrene microtiter plates (Sigma Aldrich, USA). Following incubation at 37°C for 24 hours period (Figure 3.3), the wells were washed with 200 µL PBS then dried in an inverted position for one hour at room temperature. Afterword, the microtiter plate wells were stained using (1%) of crystal violet (CV) for (15) minutes at room temperature (RT). Then, the excess stain was washed off with PBS, after that, 200 µL of 80:20 (v/v) of ethyl alcohol/acetone was used to remove CV from attaching bacterial cells. The microplate ELISA reader (BioRad, USA) was used to read the OD of the wells at 570 nm (OD570) [22]. The triplicate were applied for each test, and the bacterial strains were divided into the following divisions: influential biofilm producer, moderate biofilm producer, weak biofilm producer, or no biofilm producer [21]. For this, it was necessary to establish the cutoff value (ODc).

The ODc defines as three standard deviations (SD) above the mean OD of the negative control (un-inoculated medium): ODc = average OD of negative control + (3×SD of negative control). ***Based on the OD values:***

$OD \le ODc$ = non-biofilm producers;

$ODc < OD \le 2 \times ODc$ = weak biofilm producer;

$2 \times ODc < OD \le 4 \times ODc$ =moderate biofilm producers;

$4 \times ODc < OD$ =strong biofilm producers

RESULTS

Antibiotic susceptibility and Multiple Antibiotic Resistance (MAR) index:

Streptococcus spp. were isolated from dairy products (milk, cheese, and cream) samples collected from different dairy shops, and supermarkets in Diwanyah city, Iraq. The *Streptococcus* spp. were isolated and identified as previously mentioned in materials and methods. The antibiotic resistance profile of 42 isolates from milk (7 strains), cheese (17 isolates), and cream (18 isolates) is described in (Figure 1). High resistance was observed against novobiocin, Nalidixic acid, streptomycin, and cephalothin. However, all isolates showed a high sensitivity to vancomycin, while various level of resistance was observed for the other antibiotics tested. The mean MAR index in milk, cheese, and cream isolates was 0.34, 0.60, and 0.61, respectively (Figure 2).

The ability of bacteria to attach to various surfaces and form a biofilm [10, 11], this biofilm could contaminate the dairy products [9]. The

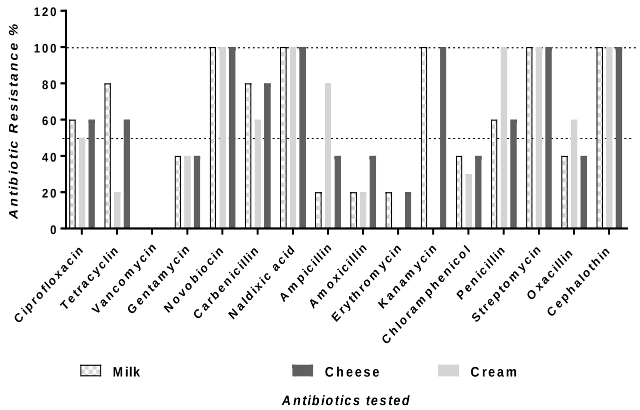

Figure 1. Antimicrobial sensitivity test results for *Streptococcus* spp. isolated from milk, cheese, and cream samples.

Streptococcus isolates showed a different level of biofilm formation with high percentages (71.43%) showed strong biofilm formation in milk isolates. While (28.57%) showed moderate biofilm formation (Table 1).

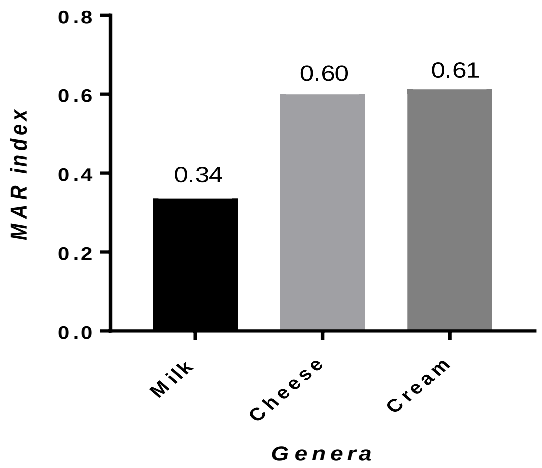

Figure 2. Multiple antibiotic resistance index of isolated *Streptococcus* from milk, cheese, and cream.

Table 1. Average of biofilm formation of *Streptococcus* spp isolated from milk samples.

Isolates	AVR	SD	Biofilm
CONTROL -	0.260	0.084	Non
CONTROL +	2.521	0.280	Strong
M6 (*Streptococcus* sp.)	2.364	0.143	Strong
M12 (*Streptococcus* sp.)	2.454	0.158	Strong
M15 (*Streptococcus* sp.)	0.844	0.109	Moderate
M18 (*Streptococcus* sp.)	2.196	0.118	Strong
M31 (*Streptococcus* sp.)	2.302	0.165	Strong
M33 (*Streptococcus* sp.)	1.397	0.130	Moderate
M37 (*Streptococcus* sp.)	2.299	0.085	Strong

M: milk and the number present the number of isolates. AVG: average, SD: standard deviation.

29.4% of *Streptococcus* isolated from cheese showed strong biofilm formation, and 52.9% showed moderate biofilm formation, while 17.7% of isolates showed weak biofilm formation (Table 2).

Table 2. Average of biofilm formation of *Streptococcus* spp isolated from cheese samples.

Isolates	**AVG**	**SD**	**Biofilm**
CONTROL -	0.260	0.084	non
CONTROL +	2.638	0.156	Strong
C1 (*Streptococcus* sp.)	1.663	0.118	Strong
C12 (*Streptococcus* sp.)	2.033	0.473	Strong
C42 (*Streptococcus* sp.)	0.886	0.048	Moderate
C50 (*Streptococcus* sp.)	1.706	0.255	Strong
C51 (*Streptococcus* sp.)	1.063	0.123	Moderate
C60 (*Streptococcus* sp.)	1.197	0.182	Moderate
C62 (*Streptococcus* sp.)	0.792	0.074	Moderate
C64 (*Streptococcus* sp.)	0.574	0.119	Weak
C65 (*Streptococcus* sp.)	0.768	0.086	Moderate
C68 (*Streptococcus* sp.)	0.910	0.179	Moderate
C69 (*Streptococcus* sp.)	0.871	0.108	Moderate
C71 (*Streptococcus* sp.)	0.619	0.075	Weak
C73 (*Streptococcus* sp.)	0.429	0.039	Weak
C74 (*Streptococcus* sp.)	1.157	0.101	Moderate
C77 (*Streptococcus* sp.)	1.762	0.104	Strong
C83 (*Streptococcus* sp.)	2.202	0.102	Strong
C84 (*Streptococcus* sp.)	0.896	0.087	Moderate

C: Cheese and the number present the number of isolates, AVG: average, SD: standard deviation.

Streptococcus isolated from cream samples showed a various level of biofilm formation with about 50% potent biofilm formation isolates, and 33.3% of isolates that isolated from cream samples showed moderate biofilm formation. At the same time, 16.7% of cream *streptococcus isolates* showed weak biofilm formation (Table 3).

Table 3. Average of biofilm formation of *Streptococcus* spp. isolated from cream samples.

Isolates	**AVG**	**SD**	**biofilm**
CONTROL -	0.260	0.084	non
CONTROL +	2.521	0.280	Strong
S11 (*Streptococcus* sp.)	0.896	0.105	Moderate
S12 (*Streptococcus* sp.)	2.533	0.258	Strong
S42 (*Streptococcus* sp.)	0.853	0.051	Moderate
S50 (*Streptococcus* sp.)	0.906	0.163	Moderate
S61 (*Streptococcus* sp.)	0.910	0.109	Moderate
S64 (*Streptococcus* sp.)	2.707	0.113	Strong
S71 (*Streptococcus* sp.)	0.685	0.069	Weak
S73 (*Streptococcus* sp.)	1.829	0.105	Strong
S79 (*Streptococcus* sp.)	0.837	0.134	Moderate
S82 (*Streptococcus* sp.)	2.449	0.153	Strong
S83 (*Streptococcus* sp.)	2.346	0.133	Strong
S108 (*Streptococcus* sp.)	2.454	0.158	Strong
S112 (*Streptococcus* sp.)	1.506	0.071	Strong
S118 (*Streptococcus* sp.)	2.499	0.136	Strong
S121 (*Streptococcus* sp.)	0.637	0.075	Weak
S125 (*Streptococcus* sp.)	2.514	0.152	Strong
S128 (*Streptococcus* sp.)	1.927	0.129	Strong
S135 (*Streptococcus* sp.)	1.128	0.151	Moderate

S: cream and the number present the number of isolates, AVG: average, SD: standard deviation.

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DISCUSSION

Streptococcus spp. are involved in many human and animal diseases [5], particularly in multidrug resistance *Streptococcus spp*. [14]. Threat of bacteria is significantly increased with the presence of biofilm within the production process of dairy products[8]. In the current study, 42 isolates of *Streptococcus spp.* were isolated from different dairy products (Milk (7 strains), cheese (17 isolates), and cream (18 isolates). *Streptococcus thermophilus* is recognized as a widely used fermentation bacteria of dairy products, particularly yogurt and cheese [23]. *Streptococcus spp*. were isolated and identified from raw cow milk [24, 25]. It is associated with mastitis [26, 27]. A plate counting and molecular investigation stated the presence of *Streptococcus thermophilus* in cheese made from buffalo cheese throughout all production processes to the storage of the product [13]. Seventeen and eighteen *streptococcus i*solate were isolated from cheese and cream, respectively. It has been indicated that the *Streptococcus* spp. exist in high concentration in dairy processing utensils and vats [28]. It has been shown that *Streptococcus thermophilus* was detected using real-time PCR throughout cheese [29].

High resistance was observed in all three products against novobiocin, Nalidixic acid, streptomycin, and cephalothin. However, all isolates showed high sensitivity to vancomycin, try to write why what advantage of this antibiotic to be effective while the various level of resistance was observed in other antibiotics tested. The mean MAR index in milk, cheese, and cream isolates was 0.34, 0.60, and 0.61, respectively (Figure 2). The non-heat treated dairy products are considered as the primary source of antimicrobial resistance bacteria transmission from animals to humans via dairy products consumption and that linked to human gastroenteritis [30]. It has been reported that *S. thermophilus* isolated from raw milk showed resistance streptomycin, neomycin [31], erythromycin, clindamycin, and tetracycline [32]. This resistance could be linked to commonly used antibiotics to treat mastitis [31]. Compared with previously reported resistance, less resistance has been reported from isolated Streptococcus to vancomycin [33]. High resistance to novobiocin could be related to commonly used novobiocin to treat mastitis either alone or with combination with penicillin [34, 35]. In contrast to the high resistance of all isolates to cephalothin and about 50% of isolates resist oxacillin, some reports showed all dairy cows' *Streptococcus* isolates were susceptible to cephalothin, oxacillin [36]

Notwithstanding the impact of *Streptococcus* contamination on a health issue, the microbial biofilm that forms on the food processing equipment leads to damage to theses equipment and causing a contamination threat of processed foods planet, particularly in dairy products that could be potential opportunistic and food bourn pathogen [37]. The biofilm formation in milk was at a high percentage (71.43%) of strong biofilm formation, and (28.57%) showed moderate biofilm formation (Table 1). In cheese, the results showed that about 29.4% and 52.9% of Streptococcus isolates from cheese presented a strong and moderate biofilm formation, respectively (Table 2). While in cream, the Streptococcus isolates showed about 50% and 33.3% strong and moderate biofilm formation, respectively (Table 3).

The Streptococcus isolated from cream sample showed various level of biofilm formation with While 16.7% of cream streptococcus isolates showed weak biofilm formation, (Table 3). The ability of bacteria to attach in various surfaces and form a biofilm [10, 11], this biofilm could contaminate the dairy products [9]. The Streptococcus isolates showed a different level of biofilm formation with high percentages (71.43%) showed strong biofilm formation in milk isolates. While (28.57%) showed a moderate biofilm formation (see Table 1). The biofilm formation is due to the ability of the bacteria to attach in different surfaces and contaminate the dairy products [9-11, 37], and the bacterial surface proteins enhance this attachment [30].

Variant level of biofilm formation has been observed in isolated *Streptococcus* spp. The high percentages showed in bacteria isolated from milk (71.43%) compared with cream (50%) and Cheese (29.4 %) of strong biofilm formation in milk (isolates showed a variant level of biofilm formation with high percentages isolates, 29.4% in cheese, and 50% in cream. It has been reported that the aggregation of *Streptococcus thermophilus* was at a high-level liquid medium [38]. And the formation of biofilm on stainless steel is influenced by the existence of milk proteins. [38] lower percentages of biofilm formation in cheese compared with milk and cream could happen due the cheese manufacturing process and pasteurization steps were reduced biofilm formation [39].

CONCLUSION

The multidrug resistance and biofilm formation have increased threat the dairy production and consequently affected human health. Theses obtained finding could help to investigate the possible risks in the dairy industry to apply effective control measures to overcome the dairy products contamination and eliminate economic losses.

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