10.21608/avmj.2025.315675.1369 Assiut University web-site: www.aun.edu.eg

COMPETITIVE INHIBITION OF PROBIOTICS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Received: 25 August 2024; **Accepted:** 30 October 2024

ABSTRACT

Probiotics are beneficial for digestive system health; while methicillin-resistant *Staphylococcus aureus* (MRSA) is considered one of the most important causes of diarrhoea in developing countries. Therefore, the current study aimed to evaluate of antagonistic effect of probiotics against MRSA, thus, 100 samples of dairy products were collected from different local markets in Assiut City, Egypt, during the period from March 2022 to June 2023. The samples were divided into Baladi yoghurt, commercial yoghurt, Baladi Rayeb, commercial Rayeb and Ras cheese (each 20). The samples were placed into sterile plastic containers and transferred to the laboratory for isolation of probiotics and methicillin-resistant *Staphylococcus aureus* (MRSA). The antagonistic activity of probiotics supernatants against MRSA was investigated using the well diffusion agar method. The obtained results revealed that *Lactobacilli* species were isolated from 30% of the Baladi yoghurt samples, 30% of the commercial yoghurt samples, 50% of the Baladi Rayeb samples, 5% of the commercial Rayeb samples and 90% of the Ras cheese samples. While the MRSA was isolated only from the Ras cheese samples at a percentage of 5%. Regarding the antagonistic activity, *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus casei* and *Lactococcus cremoris* strains showed an inhibitory effect against MRSA, while, MRSA was resistant to *Lactobacillus acidophilus* and *Streptococcus thermophiles*. In conclusion, supplementation of yoghurt with lactobacilli probiotics enhances quality and safety and increases Resistance against MRSA infection.

Key words: Probiotic, MRSA, Yoghurt, Rayeb, Ras cheese.

INTRODUCTION

Probiotics are live bacteria that are added to food and have positive health effects by restoring the microbial balance in the gastrointestinal system (FAO/WHO, 2006; Hassanzadazar *et al*., 2012). The common probiotic lactic acid bacteria (LAB) are classified into several primary genera, including *Leuconostoc*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Pediococcus*, and *Streptococcus* (Tafvizi *et al*., 2012). These bacteria increase the growth of helpful microorganisms and decrease the population

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mechanisms of pathogens, which lowers the incidence of intestinal disorders (Hawaz, 2014).

Probiotic food consumption has increased across North America, Europe, and Asia. A large number of stores worldwide carry these goods (Rufter, 2002; Çaglar *et al*., 2005). There are numerous therapeutic applications for these microorganisms and their metabolites. According to the studies, these bacteria have a significant role in inhibiting food-poising bacteria such as *Staphylococci*.

There are around 30 species of *Staphylococci*, of which 18 have the potential to cause food poisoning through the synthesis of enterotoxins, heat-stable nuclease enzyme, or coagulase. Most strains of *Staphylococcus aureus* subsp. *aureus* are common enterotoxigenic bacteria that are coagulase-positive (Le Loir *et al*., 2003; Loncarevic *et al*., 2005). For a considerable amount of time, *Staphylococcus aureus*, a prevalent bacteria linked to hospital-acquired diseases as well as community infections has been regarded as the most significant food-borne pathogen (Morandi *et al*., 2007; Pesavento *et al*., 2007).

Many different multi-drug resistant genes are present in the majority of *Staphylococcus aureus* plasmids (Graham *et al*., 2005; Ikeagwu *et al*., 2008). One of the most common infections seen in hospitals is called "Hospital-associated methicillin-resistant *Staphylococcus aureus*" (HA-MRSA). On the other hand, MRSA has been known to target healthy individuals who have come into contact with it from communities known as "Community-associated *Staphylococcus aureus*" (CA-MRSA) (CDC, 2008).

Alternative therapies are required because of the growing resistance to antibiotics and their negative effects. Evaluating probiotic bacteria's antagonistic efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) was the aim of the current investigation.

MATERIALS AND METHODS

I) Sampling:

One hundred samples of dairy products were collected from different local markets in Assiut City, Egypt, during the period from March 2022 to June 2023. The samples were divided into Baladi yoghurt, commercial yoghurt, Baladi Rayeb, commercial Rayeb and Ras cheese (each 20). The samples were placed into sterile plastic containers and transferred to the laboratory for isolation of probiotics and methicillin-resistant *Staphylococcus aureus* (MRSA).

II) Isolation of probiotic:

The enhancement of probiotic bacteria was done according to de Man *et al*. (1960) using de Man Rogosa Sharp (MRS) broth medium, and the incubation was for 48 h at 37° C in a CO_2 incubator (anaerobic condition). One loopful from the enriched broth culture was swabbed on MRS agar plates and incubated for 48-72 h at 37° C in a CO_2 incubator. The suspected single colonies were isolated and identified by using morphological, biochemical (Bergey *et al*., 1994; MacFaddin, 2000) and molecular tests according to Markiewicz and Biedrzycka (2005).

III) Isolation of MRSA:

The enrichment of *Staphylococcus aureus* was done using trypticase soy broth (Difco) supplemented with 70 mg of NaCl / ml and incubated at 35° C for 20 h (Lee, 2003). One loopful from the enhanced broth culture was streaked on mannitol salt agar (Oxoid) and incubated at 37° C for 24 h. Gram stain and biochemical tests (catalase, oxidase, coagulase, hemolysis tests) were performed on the isolates (Cheesbrough, 2006). The isolates were further sub-cultured onto oxacillin-resistance screening agar base (ORSAB, Oxoid) and incubated at 37° C for 18 h.

The suspected MRSA isolates were subjected to antibiotic susceptibility to determine methicillin resistance by a manual disc diffusion technique by means of Müller-Hinton agar following the Clinical and Laboratory Standard Institute guidelines (CLSI, 2007). The antimicrobials impregnated discs were doxycycline (30 µg), amikacin (30 µg), amoxicillin (10 µg), azithromycin (15 µg), cefoxitin (30 µg) and clarithromycin (15 µg). Additionally, the Vitek 2 system (bioMe´rieux, Lyon, France) was utilized to identify clinically significant isolates of *Staphylococci*, detect oxacillin resistance, and test for susceptibility to additional anti-*Staphylococcal* antibiotics. The cards for Gram-positive identification (ID-GPC) and susceptibility testing (AST-P507) were identified using this system.

IV) Antimicrobial activity of probiotics against MRSA:

The zone of inhibition and zone index were determined using Kirby Bauer's diffusion method for probiotic CFC (cell-free culture/supernatant) against several human infections. The antimicrobial activity of probiotic strains against the isolated MRSA was carried out according to Chuayana *et al*. (2003).

IV-a) Preparation of probiotic cell-free supernatant (CFS):

Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei, Streptococcus thermophiles and *Lactococcus cremoris* strains were cultured separately in MRS broth and incubated at 37 °C for 24 h; then, the bacterial cell-free supernatant (CFS) for each strain was prepared by cold centrifugation (4° C) for 25 min at 3500 rpm (Nowroozi and Mirzaii, 2004).

IV-b) Well-diffusion agar method (Weese *et al*., 2004):

Two ml of 10^8 of MRSA concentration using Densi Check (BioMerieux, France) was used to soft Müller-Hinton agar plates containing 20 ml medium. Following a short time of drying at 37 °C in the incubator, wells were created in each agar plate using sterile micropipette tips. Separate wells were filled with 50–200 µL of probiotic cell-free supernatants (CFS), and as a control, sterile saline was added.

The plate was incubated for 24 h at 37° C. The probiotic's ability to suppress bacteria was indicated by the inhibition zone surrounding the well, which was then measured (Vélez *et al*., 2007). Every test was administered twice.

RESULTS

Table 1: Incidence of the positive examined samples for probiotics and MRSA.

The examined bacteria	Yoghurt				Rayeb				Ras cheese	
	Baladi		Commercial		Baladi		Commercial			
	No. (20)	%	No. (20)	%	No. (20)	%	No. (20)	%	No. (20)	%
Lactobacilli	6	30	6	30	10	50	1	5	18	90
Staphylococcus aureus	1	5	2	10	-	-	1	5	17	85
MRSA	-	-	-	-	-	-	-	-	1	5

Table 2: Sensitivity test of some probiotics strains against MRSA.

MRSA (conc.)	Inhibition zone (mm)					
	Lactobacillus bulgaricus	***Lactobacillus plantarum***	***Lactobacillus acidophilus***	***Lactobacillus casei***	***Streptococcus thermophilus***	***Lactococcus cremoris***
10^6	17	24	R	16	R	16
	29	35	R	28	R	17
	18	19	R	17	R	32
10^8	18	19	R	R	R	21
	R*	26	R	R	R	22
	R	R	R	22	R	R

* Resistant.

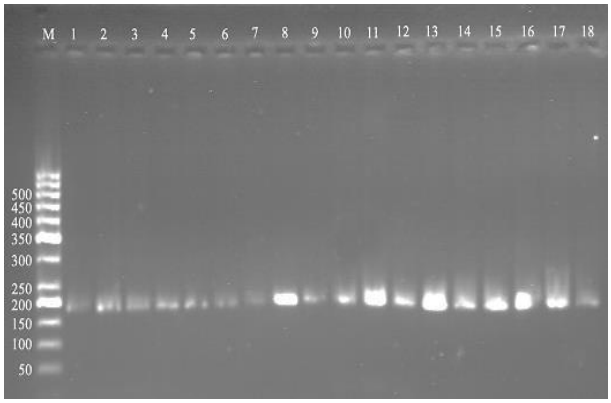

Fig. 1: PCR amplification products of the examined probiotic isolates.
Lane M: ladder. Lanes from 1 to 18: +ve for *Lactobacilli* at 232 bp.

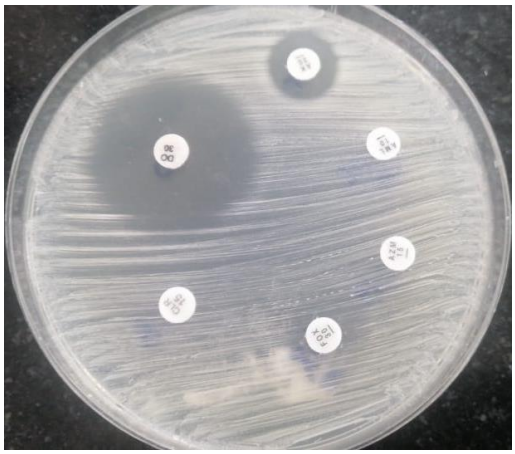

Fig. 2: Antibiotic susceptibility test of MRSA against antimicrobials impregnated discs.
DO = doxycycline (30 µg), AK = amikacin (30 µg), AML = amoxicillin (10 µg), AZM = azithromycin (15 µg), FOX = cefoxitin (30 µg), CLR = clarithromycin (15 µg).

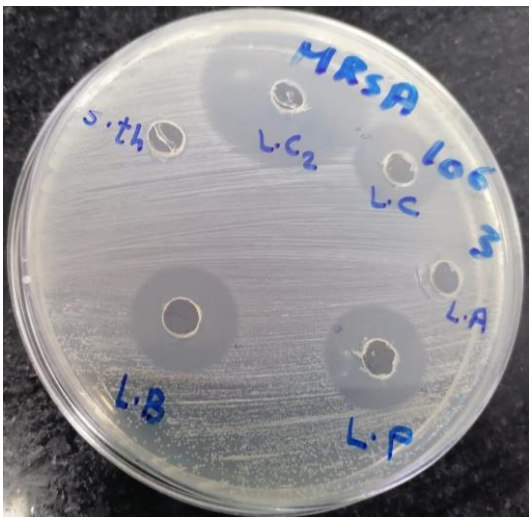

Fig. 3: Clear zones around the wells loaded with *Lactobacillus* cell-free supernatant representing the inhibiting activity of *lactobacillus* strains against MRSA.
L.B = *Lactobacillus bulgaricus*, L.P = *Lactobacillus plantarum*, L.A = *Lactobacillus acidophilus*, L.c = *Lactobacillus casei*, S.th = *Streptococcus thermophiles* and L.c2 = *Lactococcus cremoris*.

DISCUSSION

According to the obtained results in Table (1), *Lactobacilli* were isolated from the examined samples of Baladi yoghurt, commercial yoghurt, Baladi Rayeb, commercial Rayeb and Ras cheese, in percentages of 30, 30, 50, 5 and 90%, respectively. The isolated probiotic in the

current study was *Lactobacilli* which agreed with Mannan *et al*. (2017). The highest incidence of the isolated *Lactobacilli* was from the Ras cheese samples (Fig. 1). El-Shafei *et al*. (2002) and Abd El Gawad *et al*. (2010) identified a higher proportion of *Lactobacilli* from milk products.

It was clear from Table 1 and Fig. 2 that the identified MRSA was found only in the Ras cheese samples in a percentage of 5%, while the other samples were MRSA-free. Usman and Mustapha (2016) detected MRSA in 2.86% of yoghurt samples. Increasing the antimicrobial properties of yoghurt may be due to lactic acid production, lowering in pH and also other antimicrobial compounds that may be present in the yoghurt (Hassan *et al*., 2013).

The supernatant of probiotic bacteria indicated antimicrobial activities against MRSA as declared in Table 2, in which *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus casei* and *Lactococcus cremoris* strains showed inhibitory effect against MRSA, while, MRSA was resistant to *Lactobacillus acidophilus* and *Streptococcus thermophiles*. Moreover, *Lactobacillus casei* had the lowest activity when compared with *Lactobacillus plantarum*, *Lactobacillus bulgaricus* and *Lactococcus cremoris* (Fig. 3).

Based on the previously described outcomes presented in Table (2) and Fig. (3), the examined probiotic had competitive inhibition against MRSA. Moreover, the mechanism of lactic acid bacteria "LAB" in the limiting of microbial and pathogens growth is their production of lactic acid in addition to other antimicrobial chemicals that inhibit the proliferation of pathogens and microorganisms (Tadesse *et al*., 2005). According to the research conducted by Boris *et al.* (2001), Lactobacilli strains that were isolated from dairy products were found to have the strongest inhibitory impact on *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *E. coli*, and *Staphylococcus aureus*, with the latter showing the highest inhibitory effect.

According to Ota (1999), consuming yoghurt causes *Lactobacillus*, a probiotic, to colonize the gut, preventing enterohemorrhagic *E. coli* from colonizing. Different *Lactobacilli* culture supernatant can inhibit the growth of several pathogens, including *Salmonella*, *Shigella*, *Listeria monocytogenes*, and *Staphylococcus aureus*.

Kamel *et al*. (2021) reported that *Lactobacillus casei, Lactobacillus fermentum* and *Lactobacillus plantarum* are perfect to be used as probiotics to help in the prevention and control infections by *Staphylococcus aureus*, because they were proven to be safe with antimicrobial activity against the organism.

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التثبيط التنافسي للمعززات الحيوية ضد المكور العنقودي الذهبي المقاوم للميثيسيللن

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إن للمعززات الحيوية تأثير مفيد على صحة الجهاز الهضمي، بينما المكور العنقودي الذهبي المقاوم للميثيسيلين (MRSA) يعد أحد أهم أسباب الإسهال في البلدان النامية. لذلك كان الهدف من الدراسة الحالية هو تقييم التثبيط التنافسي للمعززات الحيوية ضد المكور العنقودي الذهبي المقاوم للميثيسيللن MRSA، حيث تم جمع ١٠٠ عينة من منتجات الألبان من الأسواق المحلية المختلفة في مدينة أسيوط، مصر، خلال الفترة من مارس ٢٠٢٢ إلى يونيو ٢٠٢٣ وقد تم تقسيم العينات إلى زبادي بلدي، زبادي تجاري، لبن رائب بلدي، لبن رائب تجاري، الجبن الرأس (٢٠ من كل نوع)، حيث وضعت العينات في حاويات بلاستيكية معقمة ونقلت إلى المعمل لعزل المعززات الحيوية وكذلك عزل MRSA، وبالإضافة إلى ذلك فقد تمت دراسة التأثير المثبط للمعززات الحيوية ضد MRSA بإستخدام طريقة Well-diffusion agar. وقد أظهرت النتائج نسب العينات المحتوية على العصيات اللبنية حيث كانت ٣٠٪ من عينات الزبادي البلدي، ٣٠٪ من عينات الزبادي التجاري، ٥٠٪ من عينات اللبن الرائب البلدي، ٥٪ من عينات اللبن الرائب التجاري و ٩٠٪ من عينات الجبن الرأس، أما بالنسبة إلى MRSA فقد تم عزلها من عينات الجبن الرأس فقط وبنسبة ٥٪. وفيما يتعلق بالنشاط المثبط للمعززات الحيوية فقد أظهرت سلالات *Lactobacillus bulgaricus* و*Lactobacillus plantarum* و*Lactobacillus casei* و*Lactococcus cremoris* تأثيرًا مثبطًا ضد MRSA، بينما كانت MRSA مقاومة لبكتيريا *Lactobacillus acidophilus* و*Streptococcus thermophiles*. في الختام، تعزيز الزبادي بمكملات البروبيوتيك الحيوية كالعصيات اللبنية تعزز الجودة والسلامة وتزيد من المقاومة ضد عدوى المكور العنقودي الذهبي المقاوم للميثيسللين.

الكلمات المفتاحية: المعززات الحيوية ، المكور العنقودي الذهبي المقاوم للميثيسيللن ، الزبادي ، اللبن الرائب ، الجبن الرأس.