Original Article

Study Inhibitory Effects Of Lactobacillus Plantarum Against Staphylococcus Aureus Methicillin Resistance

Mohammed Subhi Musa

Biology department, College of science, University of Kirkuk
Corresponding author: Mohammadsubhi@uokirkuk.edu.iq
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Abstract

The current study aimed to study effects of Lactobacillus plantarum on Staphylococcus aureus that isolated from different clinical sources(burns, wounds, urine) . The results showed the distribution rates of isolates for the sources from which bacteria were isolated. The highest percentage of burns sources was 85.7%, followed by wounds sources 83.3% and urine 60%.These isolates of S. aureus showed 100% resistance against Vancomycin, 90% against Tetracycline and Methicillin, 85% and 83% to Penicillin and Erythromycin, respectively, and 80% for both Ciprofloxacin and Oxacillin, the resistance was 75% and 74% to Novobiocin and Levofloxacin, respectively, while the rest of the antibiotics Trimethoprim / Sulfa Methoxamine recorded 51%, and Rifampicin and Ofloxacin had a resistance ratio of 38% and 5%, respectively. It was observed in this study the Lactobacillus plantarum broth culture appeared inhibitory activity against isolates of Staphylococcus. While the supernatant did not give inhibitory activity except for the isolate of Staph3.Also,it was observed that the biofilm of all methicillin-resistant S. aureus isolates using Lactobacillus plantarum broth showed significant differences compared to control. In addition, the scanning electron microscope results showed a decrease in the thickness of the biofilm after treatment with L. plantarum

Keywords: Lactobacillus plantarum, Staphylococcus aureus, Methicillin resistance

Introduction

Staphylococcus aureus is a diffuse, and highly adaptable pathogen that colonizes the skin and mucous membranes of the anterior nose, gastrointestinal tract, peritoneum, urogenital tract, and pharynx (den Heijer et al., 2013), and is the causative agent of a variety of human and animal diseases. Which has a significant impact on public health (Luzzago et al., 2014; Bitrus et al., 2018).It demonstrates the opportunistic pathogenic behaviour of Staphylococcus aureus in both humans and animals, and can cause several disorders such as suppurative dermatitis or abscesses, endocarditis, sepsis, and urinary tract infections, mastitis, meningitis, osteomyelitis, food poisoning, biofilm-related infections and septicaemia (Singh, 2017; Scudiero et al., 2020). It represents of the most common bacterial causes of skin infection. Staphylococcus aureus is responsible for folliculitis, furunculosis, impetigo, syndrome of toxic shock, etc (Saha et al., 2019).

Staphylococcus aureus resistance to penicillin appeared due to its production of the enzyme β-lactamase, where methicillin was synthesized to resist the degradation of beta-lactamase, but MRSA strains were identified that were resistant to all β-lactam antibiotics, and soon after the introduction of methicillin into clinical practice, until recently MRSA was predominantly a pathogen that causes hospital-acquired infections, but MRSA strains are now increasingly isolated from community-acquired infections as well. Recent years have caused great public health concerns and made MRSA infection more difficult for clinicians to treat (Palavecino, 2007).Staphylococcus

aureus is the most common, virulent and pathogenic type of bacteria that is easily transmitted through hospitals, and is usually resistant to a variety of antibiotics, which makes the infection difficult to treat (Cooper et al., 2014). For this reason, researchers attempt to find other types of antibiotics, including penicillin compounds, such as Methicillin, Nafcillin, and strains of methicillin-resistant cocci spread and constitute a major problem in many hospitals and health care centres (Fagade et al., 2010).

Recent studies point to probiotics as a promising option for fighting biofilms. Probiotics are live microorganisms which, when administered in sufficient quantities, are of health benefit to the host." Lactic acid bacteria which include: Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus and Leuconostoc are the dominant group of bacteria with proven probiotic effect, where Lactobacillus is more effective. This group of bacteria can grow in different habitats using diverse sources of carbon. From glucose metabolism, lactic acid bacteria are classified as fermenters, producing exclusively lactic acid, and producing many other metabolites besides lactic acid, such as ethanol and acetic acid (Carvalho et al.,2021).

Methods

Sample collection

Two hundred fifty Samples were collected from different sources (burns, wounds, and urine) of S. aureus isolates from patients admitted to Hawija Hospital / Kirkuk city.

Isolation and identification of S. aureus

The isolates of S. aureus were diagnosed microscopically and biochemically tests (Coagulase, Oxidase, Catalase, Urea test, DNase).Also , it was used API 20 to diagnosis this bacteria. Moreover , S. aureus methicillin resistance detected by using CHROMagar MRSA

Antibiotics sensitivity test of S. aureus

S. aureus isolates were cultivated bacteria on Blood Agar. The disc diffusion method for the microbial sensitivity test depended on the Kirby Bauer method and Mueller Hinton agar was performed for all S. aureus isolates to determine their antibiotic resistance (Melo et al., 2013).

Detection of biofilm formation

It was detected biofilm by using Congo red agar and the plate consisting of micro-calibration tubes (Mathur et al.,2006)

Antagonistic effect of Lactobacillus on S. aureus

Lactobacillus plantarum(L.p) was obtain from Selçuk University/Turkey .It was used to inhibition of S. aureus methicillin resistance. Where it was applied suspension and supernatant of Lactobacillus plantarum to detect using agar diffusion to detect activity against four isolates of S. aureus.

Results and discussion

Distribution of Staphylococcus aureus isolates from different sources

The results showed the distribution rates of isolates for the sources from which bacteria were isolated. The highest percentage of burns sources was 85.7%, followed by wounds sources 83.3% and urine 60% as shown in Table 1.

Source	Number samples	Percentage %
Wounds	30	83.3 (30/25)
Burn	70	85.7(60 /70)
Urine	50	60(30/50)

Table 1.The prevalence percentage of Staphylococcus aureus isolates from different sources

One study showed that out of 666 bacteria isolated from clinical samples, 133 samples (19.96%) belong to Staphylococcus aureus, 79 samples (78.95%) of them are isolated from pus and wound infections. Among the 133 samples of Staphylococcus aureus, 94 (70.64%) of Staphylococcus aureus were resistant to methicillin, as the prevalence of Staphylococcus aureus was highest in wounds and pus. S. aureus is one of the known pathogens, still one of the most common causes of infection pyogenic tissue in humans (Sapkota et al., 2019). While in other studies its prevalence was 30.4% and 14.4%, and other studies conducted globally also showed similar results (Shahi et al.,2018 ; Bhatt et al., 2014). S. aureus is a natural bacterium from the normal skin flora that can enter the body through broken skin, cuts, surgical wounds, burns and intravenous catheters where it causes purulent infections. And the percentage of its prevalence in burns and wounds, which indicates its main role in purulent soft tissues and wound infections. It was also observed that the rate of isolation of S. aureus bacteria in the pus samples was higher compared to the other samples (Shahi et al., 2018; Khanal et al., 2018). Some studies also showed that the prevalence of Staphylococcus aureus was 28.6% of hospital environments (Cheatham et al., 2019). Another study showed that S. aureus was isolated, its prevalence was 21.46% (50 samples from 233 samples) for different environments. The prevalence of hand swabs was 19.23% (Tibebu et al., 2021).

Detection of Staphylococcus aureus

The number of isolates obtained was S. aureus 130 samples out of 250 samples .The microscopic examination of the isolates showed that they are Gram-positive in pairs, and often in clusters, as they appeared in the form of short chains, and they do not contain a capsule and do not contain spores (Versalovic, 2011).The results of biochemical tests showed that all isolates of Staphylococcus aureus were positive for the tests (coagulase, oxidase, catalase, urea test and DNase)as shown figure 1 (McFaddin, 2000; Brooks et al., 2014).The diagnosis of Staphylococcus aureus was confirmed using API 20 and Vitek 2Compact.

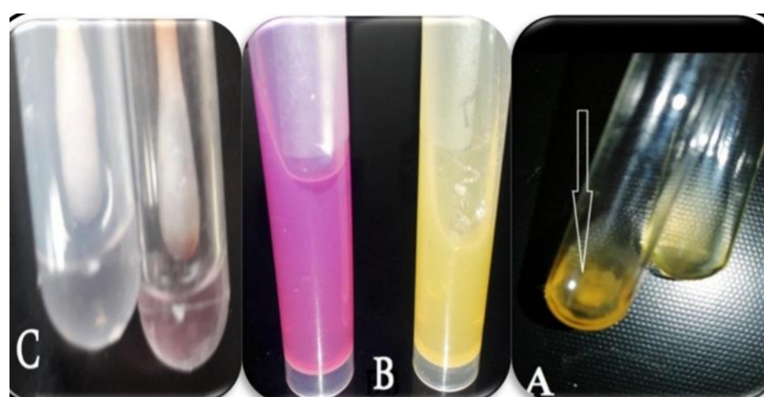

Figure1. Biochemical test of Staphylococcus aureus (A)Coagulase (B)Urea test (C) Oxidase

Figure2. Biochemical test of Staphylococcus aureus DNase test

Also,it was tested 30 isolates for their resistance to methicillin using Methicillin and all of them were resistant to Methicillin (100%) as shown in Figure 3 and 4.

Figure3. Staphylococcus aureus CHROM agar MRSA

It agreed with several studies to detect the resistance of bacteria to methicillin, one of study showed the resistance of Staphylococcus aureus isolates to Oxacillin 100% (Jahan et al., 2015). So the percentage of resistant isolates was 37.5% out of 165 samples tested (Pillai et al., 2012), and CHROM agar MRSA was also used to ensure the accuracy of diagnosing . Studies of CHROM agar MRSA for its ability to identify Methicillin-Resistant Staphylococcus aureus (MRSA), as 216 isolates of MRSA were methicillin-resistant and 241 of 226 MRSA-susceptible isolates (Diederen et al., 2005). Another used the CHROM agar MRSA, 29 isolates (25%) of the isolates diagnosed with methicillin resistance using CHROM agar MRSA medium, the sensitivity, specificity, and accuracy of CHROM agar MRSA 100%, 98.86%, and 99.13%, respectively. Thus CHROM agar MRSA could be a good, rapid and accurate selection for methicillin-resistant S. aureus (Chowdhury et al., 2014).

Antibiotic sensitivity

The results of the antibiotic sensitivity test (Figure 4) were shown against methicillin-resistant Staphylococcus aureus isolates. These isolates showed 100% resistance against Vancomycin, 90% against Tetracycline and Methicillin, 85% and 83% to Penicillin and Erythromycin, respectively, and 80% for both Ciprofloxacin and Oxacillin, the resistance was 75% and 74% to Novobiocin and Levofloxacin, respectively, while the rest of the antibiotics Trimethoprim / Sulfa Methoxamine recorded 51%, and Rifampicin and Ofloxacin had a resistance ratio of 38% and 5%, respectively.

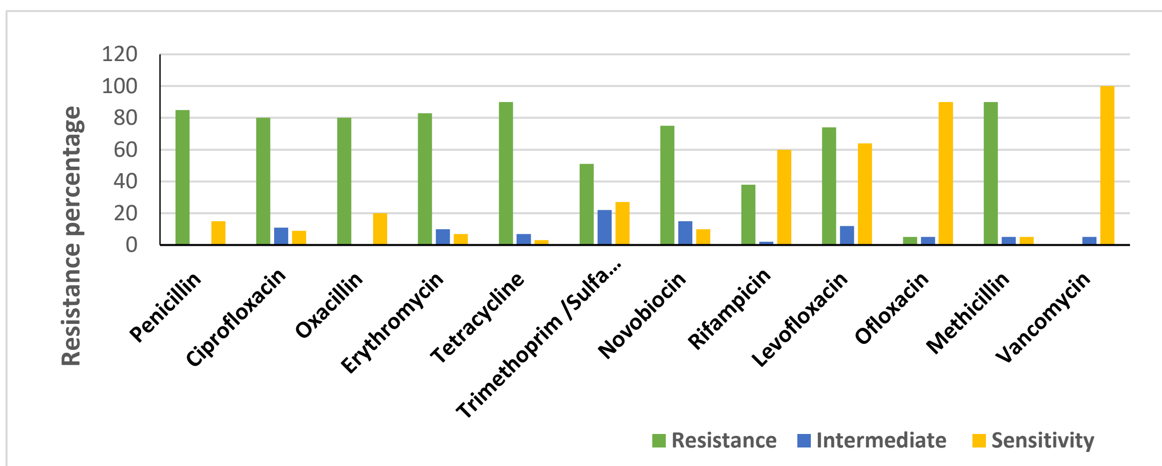

Figure 4.The percentage of antibiotics resistance against Staphylococcus aureus (MRSA)

The current study showed similar results of some studies in the resistance of methicillin-resistant Staphylococcus aureus (MRSA) isolates to some antibiotics (Gurung et al., 2020).In other studies, most S. aureus isolates were shown to be highly resistant to penicillin (71.92%), oxacillin (58.97%), and moderately resistant to tetracycline (38.49%). While it is resistant to ciprofloxacin (5.12%), chloramphenicol (2.56%), trimethoprim/sulfamethoxazole (2.56%), vancomycin (0%), and gentamicin (0%) (Bokharaei et al., 2020). In addition to another study, it was observed that S. aureus bacteria isolated from UTI patients were sensitive to Linezolid, chloramphenicol, and quinupristin/dalfopristi and resistant to nitrofurantoin, tetracycline, erythromycin and ciprofloxacin (Yousefi et al., 2016). Also, in one study, methicillin-resistant S. aureus had a 45% resistance against Trimethoprim/Sulfa Methoxamine (Sato et al., 2022). While in another study, methicillin-resistant S. aureus had the highest prevalence of resistance against penicillin (100%) , ceftaroline (100%), tetracycline (100%), gentamicin (83.33%) trimethoprim-sulfamethoxazole (80.55%) (Abdolmaleki et al., 2019). The resistance of bacteria to Rifampicin is also through the inhibition of RNA synthesis in bacteria. Thus, resistance is produced through a change in RNA polymerase as a result of a chromosomal mutation (Brooks et al., 2014). The pathogen that causes infection may vary according to its geographical spread, and therefore resistance strains must be identified and appropriate treatment found depending on the prevalence of resistance in the region (Looney et al .,2017).

Detection of biofilm formation

The biofilm of Staphylococcus aureus was detected using Congo red agar medium, where 30 methicillin-resistant isolates were tested and 80% gave positive results. A change in the color of the medium appeared from red to black as a result of biofilm production as shown in Figure (5).

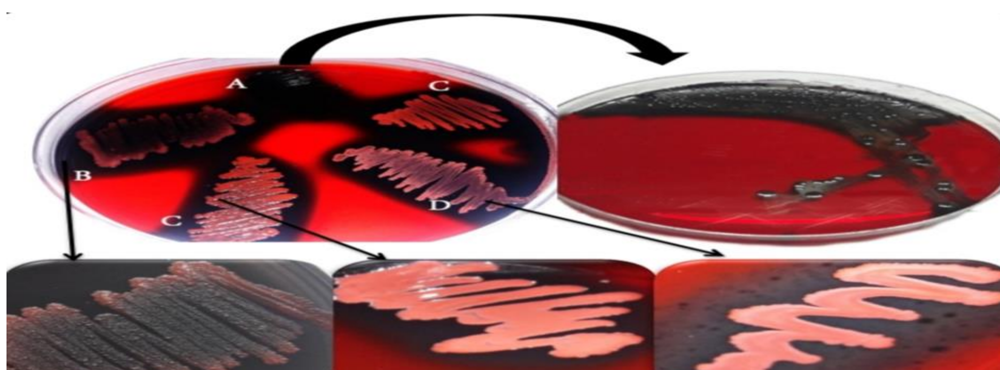

Figure 5.Biofilm formation Staphylococcus aureus

The plate consisting of micro-calibration tubes was used to detect the formation of biofilms of methicillin-resistant Staphylococcus aureus by reading the optical density using ELISA. The strong biofilm production gave a reading of optical density higher than 0.5 nm, Where 30 isolates were tested from different sources and 10 isolates were each of the sources of urine, wounds and burns. The percentages of isolates with strong production of biofilm were 70% (21 isolates), and isolates of weak production were 20% (6 isolates), and isolates of medium production were 10% (3 isolates).

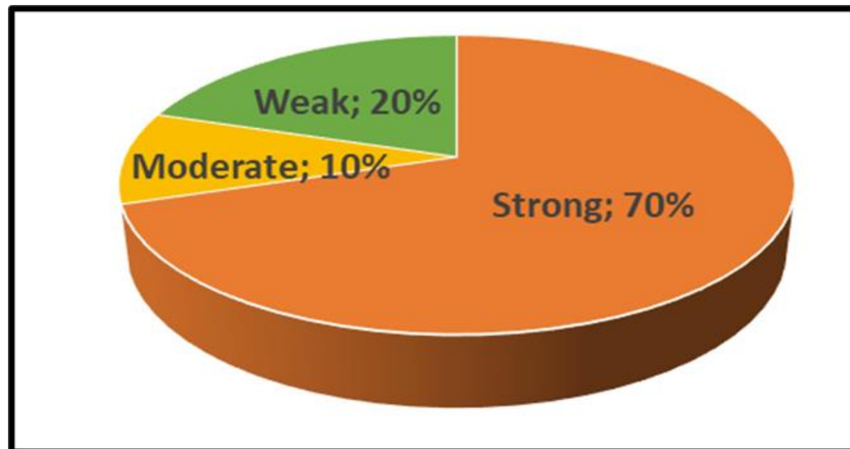

Figure 6 .The percentage of biofilm formation for different sources of Staphylococcus aureus

One study showed the ability of Staphylococcus aureus isolates to produce biofilm strongly (53%) isolated from different medical sources represented by wounds, burns, urine, pus and saliva (Abd Halim et al., 2018). Another study also recorded a high percentage of Staphylococcus aureus S. aureus bacteria produce biofilms by 64.89% (Fatima et al., 2011) and 36% (Akinkunmi and Lamikanra 2012), and another study indicated the ability of S. aureus bacteria to produce biofilms by 38%, this may be due to the difference in the sources that Bacteria strains were isolated from them (Ramakrishna et al., 2014).

Another study showed that 52.6% of the isolates of Staphylococcus aureus isolated from humans were biofilm producers. Where it was found that the clinical isolates in humans that form the biofilm are more than the coexisting isolates in animals (Achek et al., 2020). Biofilm formation is an important cause of all types of Staphylococcus associated with nosocomial infections. Biofilms produced by staphylococci isolated from other clinical samples are of clinical interest because biofilms constitute a pathogen reservoir and are associated with resistance to antimicrobial agents and chronic infections (Ramakrishna et al., 2014).

Biofilm production is one of the most important virulence factors in S. aureus because it enables it to resist or inhibit the effect of various antibiotics. The genes responsible for biofilm production were found in 50% of S. aureus biofilm producing bacteria (El-Nagdy et al. ,2020). These biofilms often serve as a source of recurrent infections. Biofilm-associated infection is a significant problem, because biofilm-associated bacteria can withstand host immune defences, and antibiotics (Hancock et al., 2010). Higher rates of multidrug resistance and methicillin resistance were found among the biofilm-producing strains compared to the non-biofilm-producing strains (Belbase et al., 2017).

Antagonistic activity test of Lactobacillus on methicillin-resistant Staphylococcus aureus (MARSA)

Lactobacillus plantarum (L.p) was tested to detect antibacterial activity against methicillin-resistant Staphylococcus aureus, and Lactobacillus supernatant and broth culture were used by agar diffusion method.

The results showed through the diameters of inhibition (Table 2 and 3) that Lactobacillus plantarum gave inhibitory activity against isolates of Staphylococcus. While the supernatant did not give inhibitory activity except for the isolate of Staph3.

Table 2. Inhibition diameters of bacterial culture of Lactobacillus plantarum towards methicillin-resistant Staphylococcus aureus isolates.

MARSA	L.p2	L.p1
Staph1	8	8
Staph2	14	9
Staph3	7	8
Staph4	9	10

Table 3. Inhibition diameters of bacterial supernatant of Lactobacillus plantarum towards methicillin-resistant Staphylococcus aureus isolates.

MARSA	L.p2	L.p1
Staph1	0	0
Staph2	0	0
Staph3	2	3
Staph4	0	0

During several studies, including Kubba (2006) noted that the inhibitory effect of Lactobacillus spp. It was higher in MRS liquid medium as it has a stimulating effect on Lactobacillus bacteria which inhibits Gram-negative and Gram-positive bacteria. It was also noted that the three isolates of Lactobacillus bacteria, L. acidophilus, L. plantarum L. casei, Where they had an inhibitory effect on bacteria with inhibition diameters of 13.6 mm, 10.3 and 10.6, as well as a mixture of these types was used and they had a strong inhibitory effect when mixed with them with inhibition diameters The highest ranges between 14.3-20.3 mm (Bhola and Bhadekar, 2019).

Also Xu et al.(2020) showed that L. casei inhibits the growth of Bacillus cereus, Staphylococcus aureus, Salmonella typhimurium and Escherichia coli. In another study, the activity of L. acidophilus, L.casei bacteria against methicillin-resistant S. aureus was observed when grown on solid and liquid media of a mixture of Lactobacillus bacteria of the species used with methicillin-resistant S. aureus, where as a result of the antagonistic reaction between them, the methicillin-resistant bacteria were eliminated with 99% after an incubation period of 24 hours at 37°C (Karska-Wysocki et al., 2010).

Antagonistic activity test of Lactobacillus on biofilm formation for methicillin-resistant Staphylococcus aureus (MARSA)

The results showed (Table 4 and 5) the antibacterial effect of Lactobacillus towards the biofilm of methicillin-resistant S. aureus isolates, as it was observed that the biofilm of all methicillin-resistant S. aureus isolates using Lactobacillus plantarum broth showed significant differences compared to isolates that were not treated with Lactobacillus bacteria. The results of using Lactobacillus supernatant also showed a decrease in the biofilm formation of S. aureus when treated with the supernatant.

Table 4. Inhibitory effect of Lactobacillus plantarum on the biofilm formation by S. aureus

Isolates of S. aureus	L. plantarum	O.D630nm Control	O.D 630nm Treatment
Staph1	L.p1	0.94±0.05a	0.310±0.2b
	L.p2	0.87±0.12a	0.315±0.1b

Staph2	L.p1	0.78±0.6a	0.520±0.3b
	L.p2	0.95±0.62a	0.330±0.5b
Staph3	L.p1	0.88±0.05a	0.590±0.2b
	L.p2	0.9±0.2a	0.300±0.4b
Staph4	L.p1	0.74±0.4a	0.266±0.09b
	L.p2	0.68±0.35a	0.240±0.7b

Different letter in rows indicated significant differences (P<0.05)

Table 5. Inhibitory effect of Lactobacillus plantarum on the biofilm formation by S. aureus

Isolates of S. aureus	L. plantarum	O.D630nm Control	O.D 630nm Treatment
Staph1	L.p1	0.66±0.06a	0.270±0.02b
	L.p2	0.7±0.4a	0.560±0.2a
Staph2	L.p1	0.3±0.6a	0.237±0.1a
	L.p2	0.54±0.7a	0.32±0.06a
Staph3	L.p1	0.52±0.09a	0.485±0.6a
	L.p2	0.53±0.08a	0.493±0.5a
Staph4	L.p1	0.51±0.15a	0.45±0.09a
	L.p2	0.64±0.5a	0.55±0.4a

Different letter in rows indicated significant differences (P<0.05)

Several studies on the susceptibility of Lactobacillus bacteria to inhibit biofilm and antagonistic of S. aureus showed that the surfactants produced by Lactobacillus acidophilus had an inhibitory effect on microbes (S. aureus, E.coli, Pseudomonas aeruginosa and Micrococcus luteus). Also, the antibacterial effect of surfactants against S. aureus depends on their concentrations (Nataraj et al.,2021). Lactobacillus bacteria contain surfactants that are produced by a variety of microorganisms with different biological functions. Whereas lactic acid bacteria were examined for their emulsifying properties. The ability of Lactobacillus plantarum to produce surface-active peptides was verified. Biosurfactant derived from L. plantarum has been shown to have the ability to reduce surface tension (Al-Seraih et al., 2022).

Conclusions

It was observed in this study the Lactobacillus plantarum broth culture appeared inhibitory activity against isolates of Staphylococcus. While the supernatant did not give inhibitory activity except for the isolate of Staph3.Also,it was observed that the biofilm of all methicillin-resistant S. aureus isolates using Lactobacillus plantarum broth showed significant differences compared to control. In addition, the scanning electron microscope results showed a decrease in the thickness of the biofilm after treatment with L. plantarum

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