MSc. Thesis

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Changes in antibiotic susceptibility of *Staphylococcus aureus* due to different stress factors

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1. **Keywords:** *Staphylococcus aureus* - antibiotic resistance – susceptibility - gamma irradiation – NaCl – enterotoxins.

2. Introduction

Microbial food safety is an essential aspect of food production and consumption, as it deals with preventing and controlling foodborne illnesses caused by harmful microorganisms. Foodborne illnesses can have serious health consequences and can be caused by various bacteria, viruses, and other pathogens. Effective control of these microorganisms requires understanding their behavior and survival mechanisms in food processing and storage environments. One of the microbes involved in food safety related issues is *Staphylococcus aureus*. *S. aureus* is a spherical bacterium commonly found on human and other animals' skin and mucous membranes. Even though many strains of *S. aureus* are harmless to their hosts, it is considered an opportunistic pathogen. Some strains can cause infections, from minor skin infections to life-threatening conditions, like sepsis and pneumonia. Nevertheless, *S. aureus* can easily invade the human body through food. Once *Staphylococcus* contaminates food, it can produce heat-resistant toxins that can cause food poisoning after consuming the food. Symptoms of staphylococcal food poisoning typically include nausea, vomiting, stomach cramps, and diarrhea which occurs within a few hours after consuming the contaminated food. *S. aureus* is also known for its ability to develop antibiotic resistance. Resistance of bacteria to antibiotics used in clinical applications has become a real threat to humanity, and the medical community has long sought a solution to this problem. Addressing the challenge of antibiotic resistance in *S. aureus* from a food safety perspective is a significant concern as well, and extensive research is necessary to identify food factors that could potentially increase or decrease bacterial susceptibility to antibiotics. It is essential to explore methods to enhance the suppressive effects of antibiotics and eliminate these pathogens at their source before they spread. While microorganisms can develop resistance to antibiotics in nature by gaining resistance genes from other species, the most prominent and common reason for antibiotic resistance is the abuse of antimicrobials. Antibiotics were discovered by pure serendipity in the

20th century and have been ever since isolated, purified, used, and developed in medicine. However, the discovery of antibiotics in 1929 did not impose its absence in human life before that date. Studies conducted on ancient nations, such as Sudanese Nubia, have confirmed the presence of antibiotics in their remaining skeletons, which are believed to date back to 350–550 CE. Another study was conducted in Dakhleh Oasis, Egypt, on skeletons of the Roman period and confirmed the same findings of antibiotics in femoral midshafts. According to these studies, tetracycline had been distributed in these population's diets.

Interestingly, these antibiotics that were present in foods were able to decrease infections as the reported infections among those populations were not high. Additionally, minimal infections were detected in bone samples. Tetracyclines are potent chelators, and this property was helpful because they were detectable in bones even after centuries. Old traditions in Jordon might give another example of the non-intentional use of antibiotics. Soils in Jordan have been used over history to date as an inexpensive alternative to pharmaceutical products. Antibiotics' presence and antibiotic-producing bacteria have been confirmed in this soil. Actinomycete bacteria which were isolated from these soils produced actinomycin C2 and C3, which are polypeptide antibiotics (Aminov, 2010). At present, the systematic development and production of antibiotics primarily led the bacteria to develop resistance to these antibiotics, and humans' efforts to combat this resistance are tirelessly ongoing.

3. Objectives

This investigation focuses on *S. aureus*, a particular type of pathogen microorganism. Antibiotic susceptibility of two strains of *S. aureus* will be compared to judge their antibiotic resistance. To achieve this, their response to ten antibiotics will be assessed. The more resilient strain will then be subjected to further examination.

- The experiment will involve exposing the bacterium to high concentrations of NaCl to determine its response.
- The bacterium will also be exposed to low dose of gamma irradiation, alone or in combination with various salt concentrations.
- The determination of surviving (culturable) bacterial cells will be conducted by spread plate method using tryptone soya agar (TSA) for each treatment.
- The alteration in antibiotic resistance will be evaluated after the treatments.
- A genotype examination will be performed to establish if there is any correlation between the effects of the treatments at genetic level.

4. Literature review

4.1. *Staphylococcus aureus* and food enterotoxication

S. aureus (*Staphylo* means grapes in Greek, and *cocci* means circular) as appears in Figure 1. is a Gram-positive, facultative anaerobic, catalase-positive, oxidase-negative, non-motile, non-spore forming bacterium. Its cells are 0.5-1.5 µm and usually form a grape-cluster structure. Its colony color is normally grey, grey-white with yellowish to orange shades depending on the conditions of growth. Majority of the bacterium possess β-hemolysis activity on blood agar. *S. aureus* is not heat stable, possessing a $D_{60°C}$ value of 1-6 minutes in foods with high water activity. The microbe is capable to invade humans via different virulence factors including its ability to form biofilms, its adhesion to the host by producing special proteins, polysaccharides, and glycans (Abril et al., 2020; Medveov & Valk, 2012). Additionally, it excretes enterotoxins that are heat-stable with D-values at 121 °C and 100 °C ranging from 9.9-11.4 to 70.0 minutes, respectively. The toxins are secreted into infected foods by the microbial cells at temperatures ranging from 10-46 °C (Ciupescu et al., 2018), and can cause lesions to the infected human cells. Furthermore, *S. aureus* can secret proteins such as coagulase, catalase, hyaluronidase, lipase, heat-resistant nuclease, staphylokinase and β-galactosidase that enable it to degrade cell components. None the less, the microbe is highly tolerant to salt concentrations up to 20% and can grow in a pH of 4.0-9.8 (Medveov & Valk, 2012) with water activity (a_w) of 0.86 (Pal et al., 2020), which imposes a serious problem if food was cross contaminated during food processing or preservation.

In 2020, the total reported outbreaks of *S. aureus* toxins in the EU were 43, where 402 people were affected, which is a considerable number of cases (EU, 2020).

Shortly after 2–8 hours from the ingestion of contaminated food, symptoms including nausea, emesis, abdominal cramps, and diarrhea will start to appear due to the Staphylococcal entero toxins (SET) (Guidi et al., 2018; Kitamoto et al., 2009). In severe cases, headache, muscle cramping and transient changes in blood pressure and pulse rate will appear (Pal et al., 2020). Regardless of these symptoms, *S. aureus* can cause infection if it penetrates blood stream, thus it can infect almost any organs, even bone tissue and cardiac valve (Medveov & Valk, 2012).

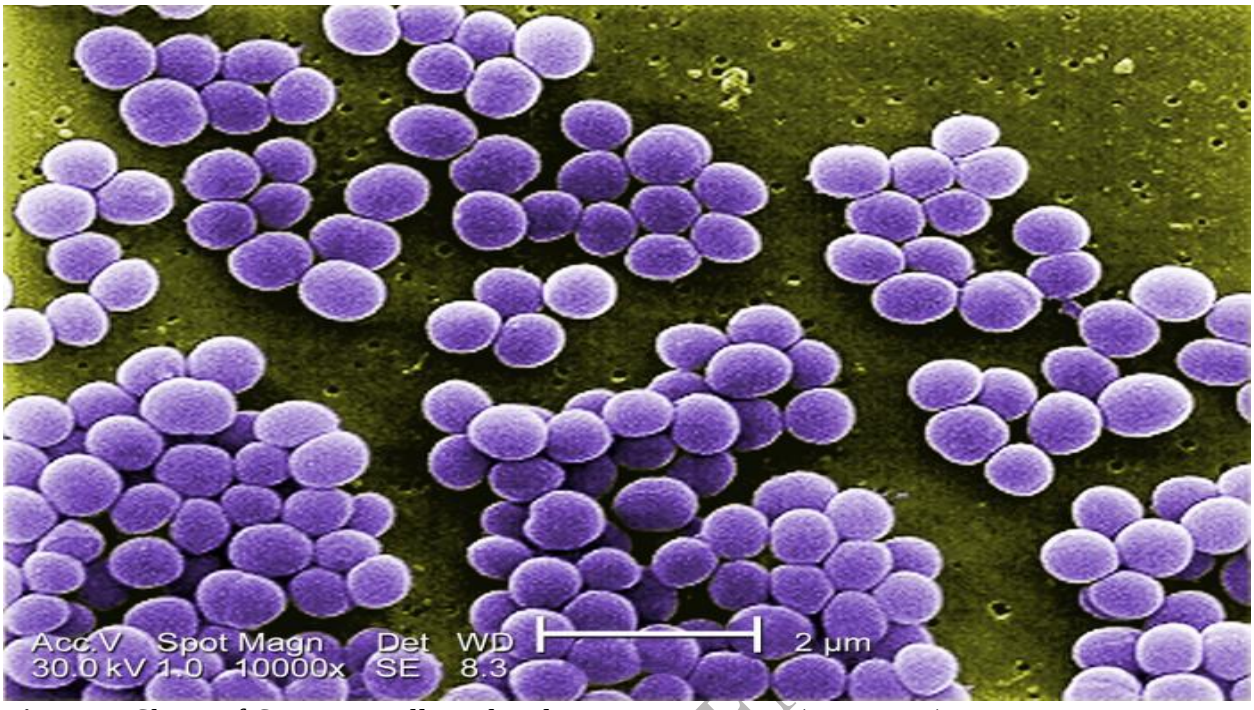


Figure 1. Shape of *S. aureus* cells under electron microscope (internet 1.)

4.2.Prevalence of *S. aureus* enterotoxins in food

Staphylococcal enterotoxins (SET) are mostly found in dairy products (especially none fully pasteurized such as some cheeses) and meat products, or in such items which were produced under non-sufficient hygienic conditions (Ciupescu et al., 2018; Medveov & Valk, 2012). In communities people consuming fresh, non-pasteurized milk could be largely affected by exotoxins. In Egypt, for instance, most of the population purchase raw, non-pasteurized milk. A recent study from Egypt reported that 18% of the total examined milk samples were infected by *S. aureus*, and 95.5% of the isolated bacteria were multidrug resistant (Elmonir et al., 2018). Source of infection can be dairy cattle as *Staphylococcus* is also a reason for bovine mastitis or could come from working staff body or nasal tract (Abril et al., 2020). Another potential food source for SET is pasta. An outbreak was reported in Italy where the pasta salad was the suspected carrier of the toxin (Guidi et al., 2018). Methicillin-resistant *S. aureus* (MRSA) was also detected in fish products due to environmental low hygiene or cross-contamination during manufacturing (Vaiyapuri et al., 2019). In Japan (2008), a university event was ended by an outbreak due to SE toxins present in crepes made by university students (Kitamoto et al., 2009)**.** Four cases out of 83 showed severe symptoms, and the patients were hospitalized and healed within days. In a recent study conducted in Greece (Papadopoulos et al., 2019), samples from the dairy industry sector

were studied. Investigation included bulk-tank milk, dairy products, employee nasal swabs and equipment surface swabs. 22% of the isolated *S. aureus* bacteria were found to be multi drug resistant, and about 3.6% were MRSA.

Bacteria can rapidly develop resistance against antibiotics because human population excessively uses these antimicrobials (Guo et al., 2020). The World Health Organization is globally collecting data since 2015 about anti-microbial resistance (AMR) due to the manifestation of antibiotic resistance issue, where *S. aureus* was considered as an important bacterium contributing to a high level of antimicrobial resistance (WHO, 2018.) In 2019, the WHO considered AMR as one of the top 10 global public health threats facing humanity, nevertheless, methicillin-resistant *S. aureus* (MRSA) is one of two main causative agents for blood stream infections (BSIs) WHO,2021).

4.3. Antibiotic resistance of *S. aureus*

4.3.1.Resistance to penicillin

It was revolutionary when antibiotics were discovered and adopted in medicine; they have treated many bacterial diseases. The first use of penicillin dates to 1941, when a policeman was admitted to a hospital in Oxford, suffering from staphylococcal and streptococcal infections in his mouth. Penicillin, discovered by chance by Alexander Fleming in 1928, was newly known to be purified at the time of the policeman incident (Gaynes, 2017), and used as an alternative to the synthetic antimicrobial drug sulphonamide, as *Staphylococcus* was already resistant to it. Although the treatment seemed successful against *S. aureus* and *Streptococcus*, the patient died as the quantity of the antibiotic produced was not adequate to treat him back then. Because of the positive results that penicillin showed in the case of that policeman, in 1944, penicillin was widely used for microbial treatment (Figure 2). At that time, most *S. aureus* isolates were susceptible by a proportion of 94%. However, resistance to penicillin in *S. aureus* occurred rapidly. By 1946, 6% of *S. aureus* produced penicillinase (an enzyme that can degrade penicillin, rendering them resistant to penicillin). In London, studies revealed a continuous increase in the percentage of penicillinase-producing isolates; in 1948, half of the strains were resistant to penicillin. Penicillinase production was found to be either plasmid-mediated or encoded by a chromosomal gene. *S. aureus* possesses one feature in producing β-lactamase that makes it a more robust organism in attacking β-lactam ring than Gram-negative organisms, because *S. aureus* produce β-lactamase external to their cell membranes, which helps protect the whole community from β-lactams. On the other hand, Gram-negative bacteria excrete the enzyme inside the bacterial

membrane. Many antibiotics discovery followed penicillin to combat resistance, such as chloramphenicol, erythromycin, streptomycin, and tetracycline. They were also effective against *S. aureus* before it became resistant, often mediated by plasmids and transposons. In the late 1950s, a clone known as phage-type 80/81 posed an international threat in many hospitals including Australia, and Japan. Use of the first cephalosporins (cephalothin and cephaloridine) began in the early 1960's as an effective solution for the penicillin-resistant *S. aureus*. None the less, gentamicin was also introduced (Gaynes, 2017).

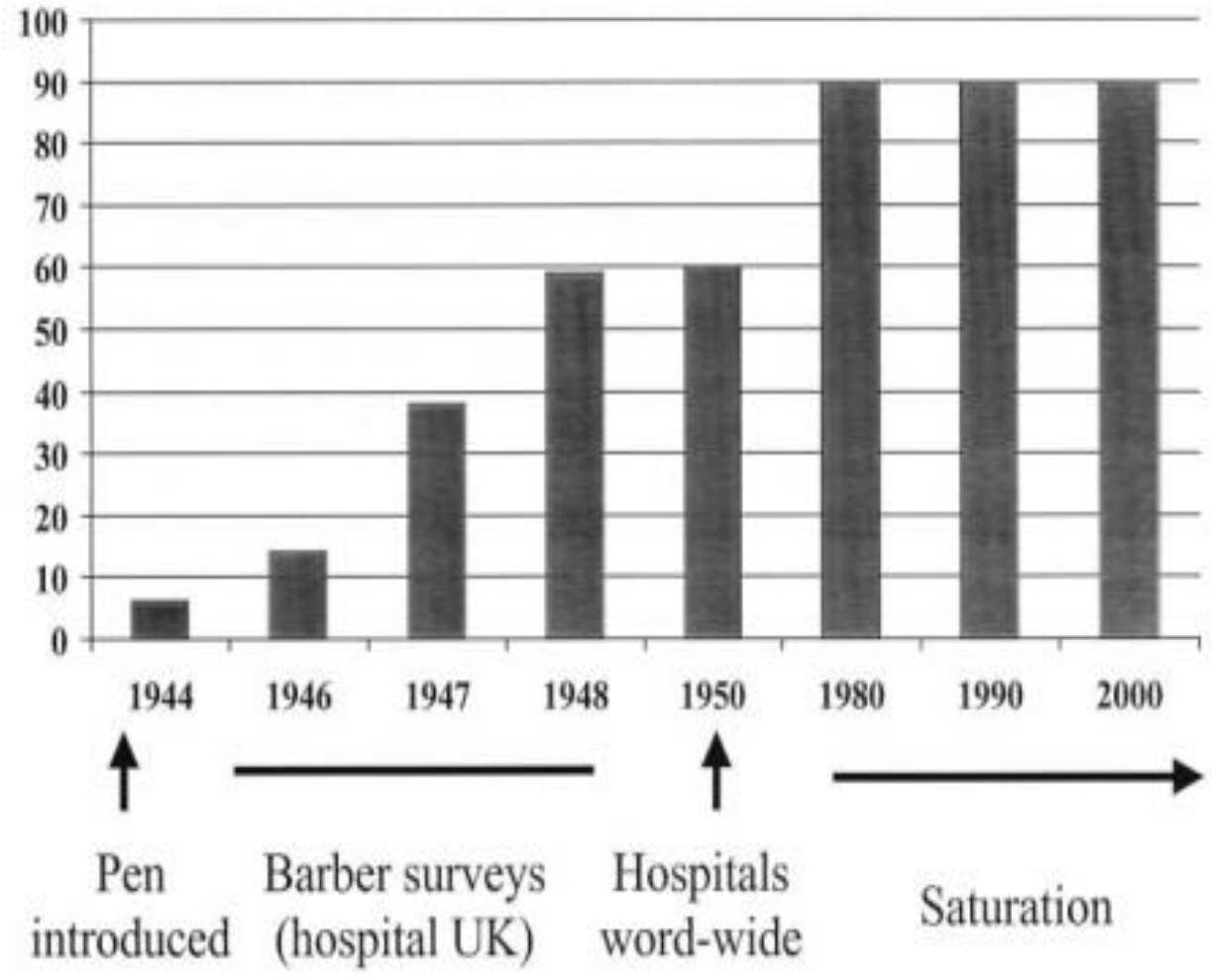


Figure 2. Acquiring of penicillin resistance **(**the accumulation of penicillinase enzyme) in *S. aureus*. Adopted from Livermore (2000)

4.3.2. Methicillin-resistant *S. aureus* (MRSA)

Continuous innovation in drugs led to modifying the penicillin structure; it originally contains phenyl acetyl group benzylpenicillin which is considered as the gold standard penicillin. The phenol group in benzylpenicillin compound was disubstituted by methoxy group (Stapleton & Taylor, 2002). Methicillin, nafcillin, and oxacillin were produced because of that achievement. These compounds have bulky 6% acyl groups that hinder the attack on the β-lactam ring by the enzyme penicillinase (Gaynes, 2017).

Methicillin-resistant *S. aureus* (MRSA) expressed its existence in 1961, the same year of the methicillin introduction. *S. aureus* originally possessed three penicillin-binding proteins, PBP1, PBP2, and PBP3. Their role is participating in bacterial cell wall building by cross-linking of peptidoglycan. The mechanism of penicillin inhibition to the microbe relies on halting the cell wall-building process. β-lactam ring has an affinity to PBPs which changes their configuration, leading to a malfunction in cross-linking of peptidoglycan. Thus, an incompletely established cell wall is formed, and the death of bacterial cells is ultimately achieved (Geisla et al, 2011.). MRSA, in contrast, has acquired the *mecA* gene, which is not found in its susceptible counterparts, imposing the hypothesis that the gene was obtained from Gram-negative bacteria then diverged to MRSA (Lowy, 2003). This gene allows antibiotic resistance by expressing an additional protein, PBP2 or PBP2a, which have low affinity to β-lactams, consequently, resist all β-lactams. Staphylococcal cassette chromosome mec (SSCmec) is a mobile element section of chromosomally inserted DNA (its size is 32–60 kb) that carries the *mecA* gene. For classifying SCCmec elements in *S. aureus* in earlier studies, there were at least three variants. These variants hold different genes, their size and organization distinguished them as types I, II, and III (Livermore, 2000). Then, SCCmec types IV to VIII were reported, and up to date, there are at least thirteen SCCmec types comprising five *mec* gene and eight *ccr* gene complexes in combination (Rezk Bottros Youssef et al., 2022).

There are differences in PBP2 production in different MRSA species due to a complex process in the gene expression. There is a cluster, *mecI-mecR1-mecA* that includes the *mecA* gene as a terminal gene. *mecI* gene includes a suppressor for the *mecA* gene, and for the latter to be expressed, the first must be deleted, or a mutation must occur. If *mecA* is successfully transcribed and translated, further enzymatic modification is done to the peptidoglycan which is regulated by chromosomal *fem* (factor essential for resistance to methicillin resistance) genes. These variation

in gene regulation between bacterial strains distinguished two different types of MRSA's PBPs production; heterogeneous, when minor cells in the community produce PBPs, or homogenous resistance when all bacterial cells produce it (Livermore, 2000).

Acquiring the *mecA* gene is due to a horizontal gene transfer because it is not self-transmissible. It is assumed that bacteriophage transduction or transpositions are responsible for the gene transfer. The percentage of MRSA was not high until the early nineteenth, when it started to rise until the late nineteenth to a proportion of 34-37% (Livermore, 2000). In the late 1990s, MRSA was still reported in hospitals (hospital-associated pathogen; HA-MRSA), then widespread to the community (community-associated MRSA; CA-MRSA) (Papadopoulos et al., 2019).

MRSA became resistant to gentamicin by the mid-1980s with a plasmid-mediated production of AAC(6')-APH(2") Ia gene (Livermore, 2000). It is a gene that encodes for an aminoglycoside acetyltransferase, a bifunctional enzyme that modifies the aminoglycoside structure of the antibiotic, thus inactivating all aminoglycosides except streptomycin (Behnood et al., 2013).

4.3.3. Vancomycin-resistant *S. aureus* (VRSA)

Vancomycin is a glycopeptide antibiotic isolated from *Streptomyces orientalis* in 1957 (Cong et al., 2020). It has been considered for MRSA treatment, however, the microbe developed resistance against it around 2002 (Guo et al., 2020). Vancomycin-resistant *S. aureus* (VRSA) was first recovered from Michigan (USA) in 2002, and 52 VRSA strains have been isolated worldwide (India, in Iran, Pakistan, Brazil, and Portugal) by that time. There are VRSA strains that are completely resistant to vancomycin nowadays, yet the occurrence of VRSA infection is still rare. Like penicillin principal mechanism, vancomycin interrupts microbial cell wall production. Vancomycin can form hydrogen bonds with a compound named D-alanyl-D-alanine (D-Ala–D-Ala) moieties of the precursor lipid II, resulting in altering its configuration. The new configuration blocks the bioprocess of transglycosylation and transpeptidation by the penicillin-binding proteins (PBPs). This alteration leads to cell wall decomposition. *Van* gene cluster (at least 11), responsible for VRSA resistance to vancomycin, was confirmed to be transferred to MRSA from *Enterococcus faecalis* in an *in vivo* study (Cong et al., 2020).

4.3.4. Current antibiotics and emerging approaches

One of the strategies used to minimize VRSA widespread is decreasing the use of vancomycin as a first-line defense by substituting it with other antibiotics, so that it becomes a last-line defense. According to the Centers for Disease Control and Prevention (CDC) recommendations, treatment

for VRSA infections should typically involve systemic antimicrobial therapy, utilizing effective antibiotics. Despite resistance to vancomycin, VRSA strains are often susceptible to various other antimicrobial agents, including ceftaroline, daptomycin, linezolid, minocycline, tigecycline, rifampin, and trimethoprim/sulfamethoxazole. According to a review (Cong et al., 2020), 90% of 13 VRSA isolates showed susceptibility to these agents. Therefore, clinicians should initiate systemic antimicrobial therapy with effective antibiotics upon identification of VRSA in clinical laboratory tests.

Quinupristin-dalfopristin and linezolid inhibit protein synthesis in Gram-positive microorganisms, including most of *in vivo* trials conducted on VRSA. However, quinupristin-dalfopristin can only have anti-staphylococcal activity when the strain is susceptible to erythromycin (Lowy, 2003). Linezolid, on the other hand, does not have cross-resistance with other antibiotics because its mechanism of action depends on binding to the 23S site of ribosomal RNA on the 50S subunit in bacteria, therefore inhibiting the 50S and 30S ribosomal subunits and preventing the formation of the 70S initiation complex, which ends by blocking protein synthesis. In 2000, the Food and Drug Administration (FDA) approved linezolid for clinical use in the US as it showed solid antibacterial effects on Gram-positive bacteria, including MRSA (Guo et al., 2020).

Another proper antibiotic in the case of VRSA, daptomycin, was obtained from the fermentation broth of *Streptomyces roseosporus*. The cyclized lipopeptide drug can destroy the electric potential of the plasma membranes in the presence of calcium ions. Daptomycin is like linezolid in the property of not having cross-resistance with other antibiotics. It can be used in treating MRSA skin and bloodstream infections, not MRSA-induced pneumonia as an exception(Guo et al., 2020).

There are many recent approaches to decrease the risk of MRSA, which do or do not include using antibiotics. These approaches may include techniques such as inhibition of quorum sensing mechanism in bacteria, decreasing the affinity of microbes to the host cells, using essential ions for bacterium to facilitate antibiotics absorbance inside the bacterial cells, use of bacteriophages in combating pathogens, and the introduction of nanomaterials (Guo et al., 2020).

Quorum sensing is a characteristic feature of bacterial communities to communicate effectively; when a particular substance (auto-inducing substances) reaches a specific level within a bacterial community, the cells start to activate specific gene expression in response(Kuo et al., 2015). These substances are excreted by bacterial cells to navigate bacterial behavior toward the production of biofilms, certain toxins, and other virulence factors for instance. The idea behind inhibiting

quorum sensing is to decrease the potential of producing virulence factors for pathogens, including MRSA. However, the narrow role of quorum-sensing inhibitors is still considered a drawback. Minimizing bacterial adherence to the host cells could be achieved by studying the agglutinating factors, such as lectin, helping the bacterial cells adhere to host cells. Lectin is a sugar that aggregates proteins and/or bacterial cells. Even though the role of lectin is shallow, medications can target designing new drugs that decrease the adherence of pathogens to their host via lectin.

Iron is an indispensable element for many enzymatic reactions ongoing in bacterial cells, so ferric ions (Fe^{3+}) can be used as a carrier for antibiotics, thereby overcoming some bacterial mechanisms to decrease antibiotic absorbance by decreasing the bacterial permeability through its outer membrane. If the ferric-antibiotic complex is present in the bacterial medium, it will penetrate inside the cell's membrane via the active transportation system; consequently, the antibiotic will be released inside the bacterial cells and start performing its antibiotic effect. The issue with this approach is that iron chelation therapy is hazardous from a toxicological perspective (Guo et al., 2020).

Another promising solution that still needs much research is bacteriophage combating antibiotic resistance. Many *in vivo* trials showed promising results; a high survival rate was obtained in infected mice due to bacteriophage therapy. Bacteriophage utilization has many benefits in contrast to antibiotics, including its high selectivity and specialty to pathogens (and not to humans), swift self-proliferation, and no cross-resistance with antibiotics. A considerable effort is still needed to address many questions in phage therapy, such as the human immune system's response to its introduction, and the fear of transferring antibiotic resistance genes via bacteriophages. In addition, bacteria can develop resistance to their phages, and the phages do not proliferate until bacterial cells achieve specific concentrations (Guo et al., 2020).

Nanotechnology is an emerging technology that can be used to address antibiotic resistance issues of many multidrug resistant microorganisms. It uses natural elements such as silver, iron oxide (Fe_3O_4), titanium di-oxide (TiO_2), zinc oxide (ZnO) and gold (Singh et al., 2014b), but with tiny particles sizes (somewhere 25 nanometers in diameter) as antibacterial drugs. China has developed a medicine targeting *Escherichia coli* and *S. aureus* effectively using this technology (Guo et al., 2020). Nanoparticles can target the issue of antibiotic resistance through many mechanisms including hindering biofilm formation, inhibition of enzyme activity, inactivation of protein synthesis, causing oxidative stress by producing free radicals, inhibition of cell wall synthesis or

causing alteration in the cell wall configuration that alters its permeability, incorporation into the bacterial DNA bases and cause destroying effect, penetration of cell membrane, and modification of essential proteins (Singh et al., 2014).

4.4.Mechanisms of antibiotics resistance in *S. aureus*

Strains of *S. aureus* differ in their susceptibility to antibiotics; some strains possess antibiotic resistance, and some are susceptible. Resistance to antibiotics can be classified into two main reasons; naturally, inherited antibiotic resistance and acquired resistance by the means of coding genes of antibiotic resistance (ABR) (Pal et al., 2020)**.**

4.4.1. Inherited antibiotic resistance

4.4.1.1.Bacterial outer membrane permeability

There are mechanisms such as decreasing the permeability of bacterial outer membrane. By application of this mode, the bacterial metabolic energy expenditure is lowered, and the absorption of surrounding antibiotics is minimalized to the lowest. Aminoglycosides are a group of antibiotics that *S. aureus* avoid by applying such technique (Guo et al., 2020). Anther mechanism is the thickening their cell wall in the presence of antibiotics that target cell wall destruction such as vancomycin. After the stressful antibiotic is removed or eliminated, studies show a recovery of cells to the original configuration (Onyango & Alreshidi, 2018).

4.4.1.2.Efflux pumping system

Efflux pumping system was firstly discovered by Ball and McMurry in the 1980 in *E. coli*, and later was found to be a natural characteristic to even sensitive bacterial species. Activation and expression of efflux system-encoding genes occurs when antimicrobial substances are present in the environment for a long-time. Efflux drug pumping system is beneficial for bacterium against all hazardous antibiotics, as it is non-selective to a specific antibiotic. Generally, there are two groups of efflux systems. One of them includes specific exporters for specific antibiotic transporters that are associated with mobile genetic elements. They can easily be interchanged between bacteria. The second system contains genes of multidrug resistance that are commonly located on the bacterial chromosome (SOARES et al, 2010). QacA is an important protein of three distinctive multi drug pumping proteins present in *S. aureus* (QacA, NorA, and Smr) which use hydrogen electron (H^+) exchange as currency of energy instead of utilizing ATP. More specifically, H^+ moves inside bacterial cells, leading to harmful substances to be depleted outside the bacterium (Guo et al., 2020).

4.4.1.3. Biofilm formation

A biofilm is a community of microorganisms characterized by cells attached to a substratum, interface, or to each other, and embedded in a matrix of extracellular polymeric substances. The cells in a biofilm exhibit an altered phenotype regarding growth, gene expression, and protein production. The biofilm is typically sessile, meaning it is fixed in place and not free-floating (Archer et al., 2011). Biofilm formation benefits *S. aureus* as a virulent factor enabling it to stabilize on the host cells. It also plays a vital role in protecting the bacterium from antimicrobial substances (Al-Mebairik et al., 2016). It can show strong resistance to antimicrobials: resistance is 1,000 times higher than in the case of planktonic counterparts (Guo et al., 2020). Two mechanisms could illustrate antibiotic resistance due to forming of biofilms on living or non-living cells. Firstly, biofilms feature the bacterial community with a lower metabolic rate, lowering the diffusion of substances to living cells. Secondly, the biofilm works as a barrier layer to restrict the penetration of some antimicrobial agents. According to Archer et al. (2011), who reported a recent study on *S. aureus* and *S. epidermidis*, the insertion ability of the antibiotic's oxacillin, cefotaxime, and vancomycin was reduced because of biofilm formation.

4.4.1.4.Persistent cells

Persistent cells have been found in biofilms, and non-biofilm-formed colonies. It is considered a natural phenomenon within bacterial populations that occurs in the presence of stressful agents such as antibiotics, apart from developing resistance genetically(Archer et al., 2011). It is defined as a subset of genetically homologous cells that behave differently phenotypically within the same microbial population. More specifically, they grow slowly and survive high concentrations of antibiotics. Keeping the state of a metabolically quiescent through a dormant form of cells enables them to protect themselves from bactericidal antibiotics(Guo et al., 2020).

4.4.2. Acquired antibiotic resistance.

Acquired resistance represents the newly gained resistance by either mutations or genetic transfer from external origin (Lowy, 2003).

4.4.2.1 Self-mutations

Self-occurring mutations have been reported in *S. aureus* (Bitrus et al., 2018): resistance to aminoglycosides, clindamycin and erythromycin are examples for such mutations (Guo et al., 2020). Another example is resistance to quinolones group of antibiotics that is essentially due to one of two mechanisms: either spontaneous chromosomal mutations in the target of the antibiotic

(topoisomerase IV or DNA gyrase), or by the induction of a multidrug efflux pump. Quinolones inhibitory mechanism depends on their effect on DNA gyrase and topoisomerase IV, which respectively reliefs DNA supercoiling and separate concatenated DNA strands. In the enzyme-DNA complex, the changes occurred because of mutations led to alteration in amino acid sequences in critical regions, known as the quinolone resistance-determining region (QRDR), consequently decreased affinity of quinolones for both their targets can be detected and cells became resistant to them. As *S. aureus* is a commensal microbe that lives naturally on the skin and nasal tract of patients, so, they are exposed to any form of antibiotic taken by patients, even for other bacteria. The most probable reason for This observation also suggests the absence of beta-lactamase enzyme and Penicillin resistance against quinolone was the usage of this drug to heal infections caused by Gram-negative bacteria. It was demonstrated that coagulase-negative staphylococcal strains gained resistance to ciprofloxacin and methicillin after treatment by ciprofloxacin. In *S. aureus,* GrlA of topoisomerase IV and the GyrA subunit in gyrase are the high likely sites of resistance mutations to occur (Lowy, 2003).

4.4.2.2. Transfer of genes

Transferring of resistance genes to *S. aureus* by horizontal gene transfer is the case for many different groups of antibiotics including β-lactams, and the location of these transmissible elements varies between plasmids, chromosome, and transposons. The class of β-lactam antibiotics comprises penicillin derivatives (penams), cephalosporins and cephamycins (cephems), monobactams, and carbapenems, which are antibiotics characterized by the presence of a beta-lactam ring in their chemical structure. As mentioned earlier, these antibiotics primarily impede cell wall biosynthesis in bacteria. Moreover, they constitute the most frequently used antibiotics in clinical practice (Bush & Bradford, 2016).

Resistance was developed to penicillin, the first used β-lactam, by acquiring the plasmids encoding for beta-lactam resistance, which is mediated by the *blaZ* gene that codes for beta-lactamase enzymes. The regulation of this gene is due to two differently transcribed genes known as *blaI* and *blaR1*. When *S. aureus* is exposed to β-lactams, the *blaZ* gene is expressed to produce the β-lactamase enzyme that degrades β-lactam. For β-lactamase synthesis to occur, BlaR1 and BlaI must be cleaved in response to the presence of β-lactams (Lowy, 2003). Methicillin, oxacillin, cloxacillin, nafcillin, and meropenem are considered β-lactam drugs but stable against β-lactamases due to a modification in their structure (Bush & Bradford, 2016). Another acquired

gene, *mecA*, was the reason for β-lactam antibiotic resistance (Bitrus et al., 2018). The regulation of the *mecA* gene is somehow like *blaZ* gene regulation. *mecI* and *mecR1* must be deleted after exposure to β-lactams so that the *mecA* gene is expressed and PBP2 is produced. Furthermore, *mecI* or *blaI* must function in all MRSA, as it is believed to be a protective mechanism to maintain a toxic protein from overproduction (Lowy, 2003).

Another horizontal gene transfer has been reported to occur in the case of VRSA. The *vanA* operon was obtained by MRSA from a vancomycin-resistant *E. faecalis.* As a result of acquiring the gene, an alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala was observed, resulting in resistance for the microbe to vancomycin (Lowy, 2003), one of the first-generation glycopeptide drugs (Zeng et al., 2016). Despite gene modifications, another mechanism of

Antibiotic	Resistance gene(s)	Gene product(s)	Mechanism(s) of resistance	Location(s)
β-Lactams	1) *blaZ*	1) β-Lactamase	1) Enzymatic hydrolysis of β-lactam nucleus	1) Pl:Tn
	2) *mecA*	2) PBP2a	2) Reduced affinity for PBP	2) C:SCC*mec*
Glycopeptides	1) Unknown (VISA)	1) Altered peptidoglycan	1) Trapping of vancomycin in the cell wall	1) C
	2)	2) D-Ala-D-Lac	2) Synthesis of dipeptide with reduced affinity for vancomycin	2) Pl:Tn
Quinolones	1) *parC*	1) ParC (or GrlA) component of topoisomerase IV	1,2) Mutations in the QRDR region, reducing affinity of enzyme-DNA complex for quinolones	1) C
	2) *gyrA* or *gyrB*	2) GyrA or GyrB components of gyrase		2) C
Aminoglycosides (e.g., gentamicin)	Aminoglycoside-modifying enzymes (e.g., *aac*, *aph*)	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides	Pl, Pl:Tn
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: *sulA*	1) Dihydropteroate synthase	1) Overproduction of *p*-aminobenzoic acid by enzyme	1) C
	2) TMP: *dfrB*	2) Dihydrofolate reductase (DHFR)	2) Reduced affinity for DHFR	2) C
Oxazolidinones	*rrn*	23S rRNA	Mutations in domain V of 23S rRNA component of the 50S ribosome. Interferes with ribosomal binding	C
Quinupristin-dalfopristin (Q-D)	1) Q: *ermA*, *ermB*, *ermC*	1) Ribosomal methylases	1) Reduce binding to the 23S ribosomal subunit	1) Pl, C
	2) D: *vat*, *vatB*	2) Acetyltransferases	2) Enzymatic modification of dalfopristin	2) Pl

Figure 3. Examples of several of the *S. aureus* mechanisms of resistance to selected antibiotics Pl, plasmid; C, chromosome; Tn, transposon; QRDR, quinolone resistance–determining region. Adopted from Lowy (2003).

resistance was observed in vancomycin intermediate *S. aureus* (VISA). Strains were characterized by the presence of increased quantities of peptidoglycan, leading to cell walls that are irregularly shaped and thickened. In addition, these strains exhibited decreased cross-linking of peptidoglycan strands, which resulted in a greater number of exposed D-Ala-D-Ala residues. This alteration in cross-linking is caused by reduced levels of L-glutamine available for the amination of D-glutamate (Lowy, 2003). Many other mechanisms are presented in Figure 3.

4.5. The use of gamma irradiation towards achieving safe food

Food irradiation can be expressed as the exposure of food to controlled amounts of ionizing radiation to improve microbiological safety and stability. Food irradiation technology is being approved by national legislations in over 55 countries worldwide for decontamination and/or sterilization of dehydrated vegetables, fruits, meats, poultry, fish, and seafood to improve product safety and shelf life (Shah et al., 2014). According to the International Atomic Energy Agency (IAEA, 2023) more than 60 countries worldwide have regulations allowing the use of irradiation for one or more food products till date.

Huge efforts have been done in the EU to ensure the safety of irradiated foods. In Geneva, September 1997, a Joint meeting for FAO/IAEA/WHO discussed the outcomes of expert committee on the wholesomeness of irradiated food (JECFI) composed in 1980. The meeting came into a conclusion that irradiation up to 10 kGy presents no toxicological hazard and introduces no special nutritional or microbiological problems. Moreover, the primary goal of using ionizing radiation in food processing is to eliminate harmful microorganisms and prevent food spoilage while maintaining the nutritional values and sensory qualities. Unlike other processing treatments, irradiation has fewer damaging effects on food properties (WHO, 1999). However, practically, the presence of irradiated foods in the EU market is still very low and undergoes strict legislations. Additionally, the acceptance of the EU consumer to irradiated food needs improvement by education and spreading informative knowledge about the safe nature of irradiated food (Farkas & Mohácsi-Farkas, 2011).

Sources for food irradiation include gamma rays, accelerated electrons and X-rays. Gamma rays are composed of high-energy electromagnetic radiation produced by the spontaneous disintegration of radionuclides. It has a strong penetrating ability (reported to be 12 cm) into foods, facilitating its use in of bulky foods. Cobalt (^{60}Co) or Cesium (^{137}Cs) are two potential sources of γ rays. Industrially, the most used source is ^{60}Co, as it provides uniform doses, it imposes low risk to the environment due to its decay into non-radioactive nickel when spent, and their high energy transfer efficiency; up to 95% of its emitted energy is available for use. ^{137}Cs, on the other hand, is concerned with safety issues and is hard to handle. Gamma rays do not give rise to neutrons, so, they are considered as safe treatments for food and do not cause food to be radioactive. A common gamma radiation setup involves placing radioactive materials such as Cobalt-60 or Cesium-137 at the top of an elevator (that can be lowered into a water pool) for irradiation. Items to be irradiated

are placed around the radioactive source at a suitable distance for the desired dosage (Munir & Federighi, 2020; Shah et al., 2014.). The unit of measurement for dose in radiation is the Gray (Gy). It indicates the amount of energy absorbed by 1 kg of a substance when 1 Joule of energy is applied. kGy/s expresses the rate at which this energy is applied. The total dose absorbed by the food being irradiated depends on the dose rate and the duration of the irradiation (Shah et al., 2014). Various irradiation doses have been described for many purposes in the case of food (Table 1).

Table 1. Different doses of irradiation and their potential applications. Adopted from Shah et al. (2014).

Dose level	**Purpose**	**Food items**
1. Low Dose Disinfestations/ delay in ripening (up to 1 kGy)	Inhibits growth of sprouts on potatoes and other foods. Kills insects and larvae in wheat, flour, fruits, and vegetables after harvesting. Slows ripening process. Kills certain harmful parasites associated with foods.	Potatoes, onions, garlic, ginger, bananas, mangoes, and certain other non-citrus fruits, cereals and pulses, dehydrated vegetables, dried fish and meat, fresh pork.
2. Medium dose Pasteurization (1-10 kGy)	Dramatically reduces number of or eliminates certain microbes and parasites that cause food to spoil. Reduces or eliminates many pathogenic microorganisms.	Fresh fish, strawberries, grape, dehydrated vegetables, fresh or frozen seafood, raw or frozen poultry and meat.
3. High Dose Sterilization (10-50 kGy)	Sterilizes food for hospital patients suffering from immune disorders and depend on diets free of bacteria. Eliminates some disease-causing viruses.	Meat, poultry, seafood and other food prepared for sterilized hospital diets, spices, enzyme preparations, natural gum.

	Decontaminates certain food additives and ingredients.	

When food matrix is irradiated by gamma (γ) irradiation, many molecules will be left ionized (in a non-stabile state) due to the excitations of electrons. Gamma irradiation affects bacteria mainly through inducing damage in proteins and macromolecules such as DNA (WHO,1999). It could be a direct effect on the nucleic acid molecules or indirect effect when free radicals are formed. Free radicals can damage the hydrogen found in the double-stranded DNA molecule. This damage can interfere with the replication process and lead to cell death. However, the impact of ionizing radiation on non-living tissues is relatively small (Shah et al., 2014.). Water radiolysis, occurs after gamma irradiation, is the main source for a following reactions of free radicals' formation that attack the nucleic acid (Munir & Federighi, 2020). It was also proposed that the crakes and pores caused by irradiation to the bacterial cells are the reasons behind the death of bacterial cells (Munir & Federighi, 2020; WHO,1999).

4.5.1. Factors influencing the D_{10} values of gamma irradiation

Munir & Federighi (2020) described D_{10} value as the value of irradiation needed for decreasing the number of surviving bacterial cells with one log/g (or 90% of the viable Colony Forming Unit (CFU)). D_{10} values determination for gamma irradiation for a food is affected by many factors. The atmosphere surrounding the microorganism is one important factor, percentage of oxygen for instance influences the overall free radicals formed because of irradiation as well as peroxide formation. The lethal dose is also dependent on the state of the food at which irradiation is performed; in frozen foods water activity is lower. Similarly, dried media offer the same effect of frozen foods regarding lethal irradiation dose. Dose rate at which irradiation is performed determines the overall outcome; higher the dose is higher the damage to the organism or material. Water activity is an important factor; the high-water activity of a medium is, the lower D_{10} value is needed. A change in the medium pH will change the lethal dose; lowering the medium pH will increase the bacterial susceptibility to irradiation. Chemical composition change of a medium will influence the overall irradiation treatment; the free radicals present will change because of the chemical composition (WHO, 1999). There are also factors that depend on the material or food being irradiated, its geometric dimensions and shape, and the packaging material used. Additional factors are related to the nature of irradiated organism and its state or phase, such as species and

cells' composition. In general, bacteria are more sensitive than fungi, and viruses are the most resistant. For example, to achieve significant reductions (3-5 log/g), doses ranging from 1 to 3 kGy should be applied in the case of fungi and higher for viruses, resulting in a deterioration of the sensory quality of some fresh and minimally processed produce. There is a difference between bacterial species in their required doses; like in the case of heat treatments, endospores of spore-forming bacteria are resistant to irradiation. Doses below 10 kGy may only cause a 2-3 log reduction in spore numbers, which may require higher doses to produce shelf-stable foods (Shah et al., 2014.). Gram-positives, including *S. aureus*, are more resistant than Gram-negatives due to differences in the cell wall structure. It was reported that 0.58 kGy is a sublethal dose for *S. aureus* which could be altered because of changing one or more of the previously mentioned factors (Munir & Federighi, 2020).

Fungus shows variations in their sensitivity to radiation, multicellular spore forming fungus including Penicillium sp., Aspergillus sp., Fusarium and Alternaria are more resistant for the fact that, if one spore survived the treatment it would re-grow again (Shah et al., 2014.).

Generally, the D_{10} values for pathogens on produce was reported to vary from 0.2–0.8 kGy (Shah et al, 2014.) and therefore, a 5-log reduction would be achievable with doses between 1 and 4 kGy. It was reported that D_{10} value for *S. aureus* can be achieved by doses ranged from 0.26 kGy to 0.57 kGy in non-frozen foods, and between 0.29 kGy to 0.95 kGy in frozen foods (Farkas & Mohácsi-Farkas, 2011). In a study on meat samples (Baradaran-Ghahfarokhi et al., 2013) doses ranged from 2 to 10 kGy, which is the maximum allowed dose by the EU regulations, contributed to decrease the viable cells in the studied food, and level of 7 kGy was sufficient to eliminate all *S. aureus* cells to non-detectable level.

It is worth mentioning that gamma irradiation is compatible in the elimination of insects and other pests in agricultural products. Although there are old techniques such as fumigation, they cause chemical hazardous imposing health and environmental risks, and are banned in a major part of the world. Doses vary depending on factors including the stage of insect life and its species. Generally, a dose of 0.7 kGy for eggs to 1 kGy for mature insects have showed accepted results in elimination of pests in different stages of life. It was also reported that a dose of 500 Gy is generally effective for most pests through their life cycles (Shah et al., 2014.).

4.5.2. Nutritional and sensorial characteristics of irradiated foods

Irradiation has emerged as a prosperous method for preserving food while minimizing disruptions to their functional, nutritional, and sensory characteristics, particularly at lower radiation doses. Nevertheless, subjecting food to high doses of radiation, exceeding 10 kGy, may cause physical and chemical transformations that can substantially impair the sensory qualities of the food, such as taste, aroma, texture, and colour. Studies indicated that when it comes to lipids, a relatively high dose of irradiation results in a milder decomposition as compared to the decomposition that takes place at normal cooking temperatures. Furthermore, when food proteins were studied, relatively low degradation occurred compared to model systems (Shah et al., 2014.). Irradiation might even not cause a significance difference in the content of nutrients, Mohácsi-Farkas et al. (2014) found that 1 kGy of gamma irradiation had no significance on the content of vitamin C and carotenoids on sliced tomatoes before and after the treatment. In another study, gamma irradiation increased the content of antioxidants like polyphenols in chestnuts (*Castanea sativa*) (Carocho et al., 2012).

4.5.3. Legislations of irradiated foods

Many countries considered irradiation as a useful technology for reducing pathogens for public health significance as part of overall good manufacturing practice (GMP) and hazard analysis of critical control points (HACCP) systems. In the United States of America, it is mandatory to set label irradiated food with “Treated with irradiation” or “Treated by irradiation” and the logo of RADURA is required for goods to be sold (Shah et al., 2014). It is a must in the EU for all irradiated foods or foods containing irradiated ingredients to be labeled (EFSA, 2023).

4.6. Salt (NaCl) and *Staphylococcus aureus*

S. aureus is an organism known with its surviving and adapting property to some of the harshest environmental factors; it possesses tolerance to high salinity up until 20%. A research has shown that biofilm produced by *S. aureus* is strongly linked with NaCl concentrations; increasing NaCl concentrations can stimulate the increased production of biofilm by upregulation of genes (such as the *icaA*) related to biofilm formation. Biofilm provides *S. aureus* with a safeguarded environment that shields the bacterial cell membrane from direct contact with salt, enabling the bacteria to continue growing in unfavorable environments (Feng et al., 2022). In the study of Feng et al. (2022) the NaCl content was increased from 0% to 10% and 20% in the media prepared for *S. aureus.* All treatments allowed the growth of the pathogen but with different patterns and different cells shapes. While bacterial cells showed integrated cell membrane and smooth cells

features that is characteristic to normal described *S. aureus* when treated with 0% sodium chloride, in presence of 20% NaCl the cytomembrane of some of the cells was ruptured, releasing the contents of the cells. In the case of 10% NaCl however, most cells presented slight change in morphology. Pattern of growth showed a gradual decrease in the growth with the increase in osmolarity for 10% and huge effect on growth was seen for 20% of salt.

Furthermore, several studies suggest that certain small molecules known as compatible solutes, including glutamine, proline, and glycine betaine, can accumulate within *S. aureus* cells due to increased biosynthesis or uptake, and reduced degradation mechanisms. This accumulation leads to an improvement in osmo-tolerance (Feng et al., 2022; Onyango & Alreshidi, 2018). In a study ofGrahamt & Wilkinson (1992) mechanisms of somnolence of *S. aureus* was investigated. Compounds of choline, L-proline, and glycine betaine demonstrated an osmo-protective effect by promoting the growth of *S. aureus* in high osmolarity conditions on various types of solid and liquid media. However, taurine was found to offer an osmo-protective effect on high-NaCl solid medium lacking other osmo-protectants. The levels of potassium pools were high and similar in cells grown at different osmolarities. Under osmotic stress, glycine betaine accumulated to high levels, and choline was converted to glycine betaine. Although proline and taurine also accumulated in response to osmotic stress, the levels were lower compared to glycine betaine.

Another study supported the same finding regarding penta-glycine residues found after stressing *S. aureus* with high saline solutions. The study examined two strains, one was isolated from fermented sea food (osmotolerant) and the other was susceptible to high salinity. To study how staphylococci tolerate osmotic stress, the obtained strains have been exposed to NaCl-rich media. Initially, the increased osmotic pressure caused *S. aureus* cells to lose water, resulting in lower turgor pressure and cell shrinkage. The sensible cells to high salt were unable to compensate for these changes, exhibited retarded growth, impaired daughter-cell separation, and abnormal cell wall characteristics was noted due to the inhibitory effects of osmotic stress. In contrast, salt-tolerant cells exhibited larger cell sizes, tetrad/cubical cell shapes, and thicker cell walls. These features were believed to help alleviate water efflux and other inhibitory effects of osmotic stress by reducing the surface area-to-volume ratio. Furthermore, penta-glycine was analyzed using high performance liquid chromatography (HPLC). The profiles of stressed cell walls showed lower amounts of penta-glycine residues in the case of the osmotolerant strain. When glycine addition is disrupted during peptidoglycan assembly due to high salinity, shorter interpeptide bridges and

fewer cross-linkages between the muropeptide layers and a reduction in the synthesis of cell wall-associated proteins are resulted. This results in a loosely linked peptidoglycan layer which is less susceptible to the hydrolases that facilitate cleavage and turnover, resulting in thicker cell wall. The peptidoglycan strands in cell wall were examined further using Solid-state nuclear magnetic resonance (NMR) technique, and it was stated that NaCl stress was not able to affect the already formed peptidoglycan strands, and the newly synthesized peptidoglycan was only affected (Onyango & Alreshidi, 2018). In another reported study by Onyango & Alreshidi) (2018), phospholipid content of the bacterial cell membrane was analyzed for *S. epidermidis* incubated in media with NaCl concentrations <15%. The fatty acid profiles of the investigated strain showed no significant change, but concentration of anteiso-fatty acid (C15:0) was elevated when the concentration was increased to 25% NaCl (Onyango & Alreshidi, 2018).

5. Methods and materials

5.1.Collection and maintenance of *S. aureus* strains

Two strains of *S. aureus* (B.01755 and B.02174) were obtained from the National Collection of Agricultural and Industrial Microorganisms, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences (Ncaim.Etk.Szie.Hu). The strains were provided on slant agar. Using sterile inoculating loops, two new parallels of each strain were sub-cultured on Tryptone Soya Agar (TSA) slants by transferring bacteria to sterile solidified TSA slant tubes. The inoculated tubes were incubated at 37 ℃ for 24 hours, then kept in the fridge at 4 ℃ for the whole study period.

5.2.Preparation of media

- For counting living bacterial cells, tryptone soya agar (TSA) was prepared by adding tryptone soya broth (Biolab Zrt. Hungary) with bacteriological agar (VWR chemicals, Leuven-Belgium), and were suspended in distilled water.
- For obtaining an optical density of 0.5 McFarland, a solution of Tryptone soya broth (TSB) was prepared to suspend the bacterial cells for measurement. The solution was prepared by adding (TSB) powder to distilled water.
- For disk diffusion antibiotic testing Mueller Hinton Agar was prepared by adding 10.5 g Mueller Hinton broth powder (Carl Roth GmbH-Germany) to 7.5 g of bacteriological agar (VWR chemicals, Leuven- Belgium) and 500 ml of distilled water.
- For serial dilution, a dilution liquid was prepared by adding 0.36 g of peptone (Biolab Zrt. Hungary) and 3.06 g of NaCl to 360 ml of distilled water.

All prepared media and solutions were autoclaved for 15 minutes at 121 ℃. The agar media were let to cool down to 50 ℃, poured into sterile plastic Petri dishes, and left to solidify.

- The antibiotics used were ready-for-use paper disks: Piperacillin 100µg, Nalidixic Acid 30µg, Erythromycin 15µg, Meropenem 10µg, Colistin 10µg, Piperacillin 30µg, Gentamycin 10µg, Ampicillin 10µg, Chloramphenicol 30µg, and Ciprofloxacin 5µg (Bio-Rad, France).

5.3.Tryptone soy agar (TSA) culturing and achieving required optical density (OD)

24-hour fresh cultures of the two *S. aureus* strains (B.01755 and B.02174) were obtained by transferring inoculum from the slant stock agars to TSA medium on Petri dishes using the streak plating method and incubated at 37 ℃. The next day using sterile plastic loops, a loopful was taken from each strain and suspended in tubes containing 5 ml of sterile TSB to have concentrations of

10^8 ml^{-1} for each strain; an optical density of 0.5 McFarland was used for that purpose. These suspensions were used as a condensed bacterial suspension to conduct the antibiotic disk diffusion test on MHA.

5.4.Antibiotic disk diffusion test

The bacterial suspension of TSB having OD of 0.5 McFarland was well vortexed. A sterile cotton swap was immersed and used for plating; the Petri dishes containing sterile solidified Mueller Hinton Agar (MHA) were rotated three times at 60° for an even distribution of inoculum. The ten antibiotics were then applied using sterile tweezers to the Petri dishes in three parallels and incubated at 37 °C for 16-18 hours (Hudzicki, 2009; *Laboratory Guide: Methodologies for Antimicrobial Susceptibility Testing APEC Sub-Committee on Standards and Conformance*, 2020). The results were compared to the table provided in Antibiotic Susceptibility Test (BBL, 2011).

5.5.Treatment of *S. aureus* B.02174 strain

The effects of two different environmental stresses were examined alone or in combination:

- gamma irradiation (0.6 kGy dose),
- osmotic stress (presence of sodium chloride in 6%, 10%, and 12%), and
- combination of gamma irradiation with osmotic pressure (6%, 10%, and 12% NaCl).

For testing the effect of these stressors, 10^8 cells/ml concentration of *S. aureus* B.02174 was obtained employing 0.5 McFarland OD and treated by the three different concentrations of saline solutions, 0.6 kGy gamma irradiation, and the third treatment by exposing the bacteria to the 0.6 irradiation dose for the salinated sample. The formerly used ten antibiotics (Piperacillin 100 µg, Nalidixic Acid 30µg, Erythromycin 15µg, Meropenem 10µg, Colistin 10µg, Piperacillin 30µg, Gentamycin 10µg, Ampicillin 10µg, Chloramphenicol 30µg, and Ciprofloxacin 5µg (Bio-Rad France) were applied on MHA to observe the alteration of bacterial susceptibility after all treatments. A control sample was used in the cases of the investigations: this sample was treated neither by irradiation nor NaCl. Number of surviving bacteria were determined to obtain information about the effects of all treatments.

Phosphate buffer saline (PBS) was prepared as the basic solute for treatments. Three various NaCl concentrations, namely 6 %, 10 %, and 12 % were set in PBS. PBS preparation for the control treatment (0% salt) was obtained by adding 0.8 g of NaCl (Thomasker Finomvegyszer Kft. Hungary), 0.02 g of KCl (Biolab Zrt. Hungary), 0.144 g of Na_2HPO_4, and 0.024 g of KH_2PO_4

(Reanal Laborvegyszer Kereskedelmi Kft. Hungary) to a volume of 100 ml distilled water in a 150 ml bottle and sterilized.

Stressing proceeded firstly with 6% NaCl concentration; 6% NaCl salt concentration was considered alone, 0.6 kGy gamma irradiation without NaCl, and a combined treatment of 6% NaCl and 0.6 kGy gamma irradiation were done. A control sample that did not contain additional NaCl salt or irradiation treatment was considered. 10 ml of each sterile PBS containing different NaCl salt concentrations (0 % and 6 %) were transferred to a sterile tube. 24-hour-old *S. aureus* B.02174 was cultured from the stock slant agar. From the 24-hour fresh culture, a loopful of bacteria were then transferred to the tubes containing 10 ml of sterile PBS of each salt concentration. The optical density was adjusted to 0.5 per bacterium suspension. Each tube was vortexed, and 5 ml of the 10 ml was transferred to another tube to have the irradiation done on one of the two parallels. Irradiation was done using a panorama type Co^{60} γ–beam source at Centre for Energy Research (Budapest). After irradiation, the samples were cultured for both antibiotic susceptibility on Muller Hinton agar and viable (surviving) cell count on TSA. Before plating on MHA, concentrated bacterial cells were obtained after irradiation by centrifugation of the samples at 12000 RPM for 5 minutes at 4 ℃. Moreover, 1 ml from each tube was transferred in a new Eppendorf tube and preserved in the lab freezer at -18 ℃ for further PCR investigations. As mentioned earlier, a cotton swap was immersed in the treated tubes and crossed against MHA for antibiotic susceptibility test. Serial dilutions have been performed for determining the viable cells on TSA. All Petri dishes were incubated at 37 ℃ for 24 hours, and the antibiotic susceptibility results were measured using a simple ruler.

After obtaining results of up to 6 % NaCl concentration, the second treatment was proposed to increase NaCl salt concentrations of 10% and 12% as individual treatments, and combined with 0.6 kGy gamma irradiation. The second trial did not include all ten antibiotics; only Meropenem (10 µg) was considered as it showed increased bacterial susceptibility after increasing osmosis and irradiation treatment. The 24-hour-old culture was prepared from the agar slant, as mentioned earlier. 10 ml of each sterile PBS containing different NaCl salt concentrations (0, 6, 10, 12%) were transferred to a sterile tube. Bacterial loopful were transferred, and the optical density was adjusted to 0.5 per bacterium suspension. Each tube was vortexed, and 5 ml of the ten was transferred to another tube to have the irradiation done on one of the two parallels. Irradiation was done as earlier. After irradiation samples were cultured for both antibiotic susceptibility on Muller

Hinton agar and total viable count on TSA. To concentrate the bacterial cells after irradiation for susceptibility testing, the samples were centrifuged at 12000 RPM for 5 minutes at 4 ℃, and similarly to the previous experiment 1 ml from each tube was transferred in a new Eppendorf tube and preserved in the lab freezer at -18 ℃ for further PCR investigations. All the other steps were the same as before.

5.6. DNA extraction and purity determination

The frozen samples in Eppendorf tubes were allowed to melt and centrifuged (12.000 rpm, for 5 min, at 4 ℃). The supernatants were carefully, under aseptic conditions, pipetted away from the bacterial cells and filtered using 0.45 µm PES (polyether sulfone) membrane filters into another sterile Eppendorf tubes (thus gaining the extracellular (e)DNA fraction of the treated samples). The cells have been resuspended in 100 µl TE buffer (10 mol dm^{-3} Tris-HCl, 1mmol dm^{-3} EDTA; PH 8). Aseptically, 0.3 g glass beads (diameter: 0.4-0.6 mm) have been added to the resuspended cells and vortexed for 3 minutes, then samples were boiled for 15 minutes and let to cool down. Centrifugation process (12,000 rpm, 5 min, 4 ℃) was followed to remove debris. 30 µl of the upper layer were transferred to new sterile Eppendorf tubes leaving the debris in bottom (this fraction was considered as intracellular (i)DNA). DNA content and purity of filtered supernatants and lysed cells have been measured using Nano Drop 2000 (Thermo Scientific). 2 µl of the same TE buffer used in extraction step was measured firstly as a base line of the measurements for blanking, then 2 µl of each sample has been measured. The samples were placed on a special sensor of the instrument and its led was closed. dsDNA analysis was chosen, and concentration was obtained in ng/µl. To judge the purity of DNA, the ratio of absorbance at 260/280 nm was used. Usually, a ratio of 1.8-2 is accepted as “pure” for DNA. The DNA samples were then stored at -18 ℃ for further analysis.

5.7. PCR test

mecA gene, responsible for beta-lactam antibiotic resistance in *S. aureus*, was amplified using the forward (5’ ACGAGTAGATGCTCAATA 3’) and reverse (5’ CTGGAATAATGACGCTATG3’) primers designed in the study of Kovács and co-workers (unpublished results). Composition of the reaction mixture was the following (Table 2):

Table 2. Composition of reaction mixture for PCR amplification of staphylococcal *mecA* gene

PCR mixture components	Quantity 1 sample	Quantity 18 sample
Buffer (5x)	5 µl	90 µl
25 mM dNTP	0.1 µl	1.8µl
10 µM OXA Forward	0.5 µl	9µl
10 µM OXA backward	0.5 µl	9µl
One Taq® DNA polymerase (new England, bio labs) (5U/µl)	0.1 µl	1.8µl
DNA sample	1 µl	18µl
Sterile distilled H_2O	17.8 µl	320.4µl
Sum	25 µl	450 µl

A 125 base-pair-long segment of the *mecA* gene was amplified. The PCR protocol was the following: 94 ℃ for 4 minutes; then 30 cycles: 94 ℃ for 30 seconds, 44 ℃ for 30 seconds, and 68 ℃ for 30 seconds; final extension at 68 ℃ for 3 minutes.

5.8. Gel preparation and electrophoresis

Agarose gel (Promega, USA) was prepared to have the DNA segments separated and detected on it. For preparation, 1,2 g of agarose powder was added to 120 ml 0.5X TBE buffer (5.4 g tris base, 2.75g boric acid, 2 ml 0.5M EDTA solution, pH 8.0), and heated in the microwave until obtaining a clear transparent solution. The agarose gel was let to cool down until 50 ℃, and 4 µL of a gel staining dye (GelStarTM Nucleic Acid Gel Stain, 10,000X) have been thoroughly mixed with the gel. The gel was then poured into a tray, and the comb has been inserted. After the gel solidification, almost 20 minutes later, the comb was carefully removed leaving wells for loading the samples, and the gel was placed into the electrophoresis chamber (filled with 0.5X TBE buffer). 1 µl loading dye (Gel Loading Dye, Blue 6X, Biolabs, New England) mixed with 5 µl of the DNA samples were loaded into the wells. Running the gel took almost 90 minutes at 90 Volts, then the electricity was disconnected, and results were observed with the help of UV transilluminator (BioRad).

5.9.Statistical analysis

One-way analysis of variance (ANOVA) test was performed to examine the significance of results using Microsoft Excel 365 (Office).

6. Results and discussion

6.1.Antibiotic susceptibility of *S. aureus* B.01755 and B.02174 strains

Bacterial susceptibility to antibiotics for the two *S. aureus* strains was compared (Figure 4). The clearing zones around the tested antibiotics were recorded for both strains, and the means were calculated (Table 3). Following this a similarity tree was constructed using PAST 4.12 software (Hammer et al., 2001) (Figure 5.).

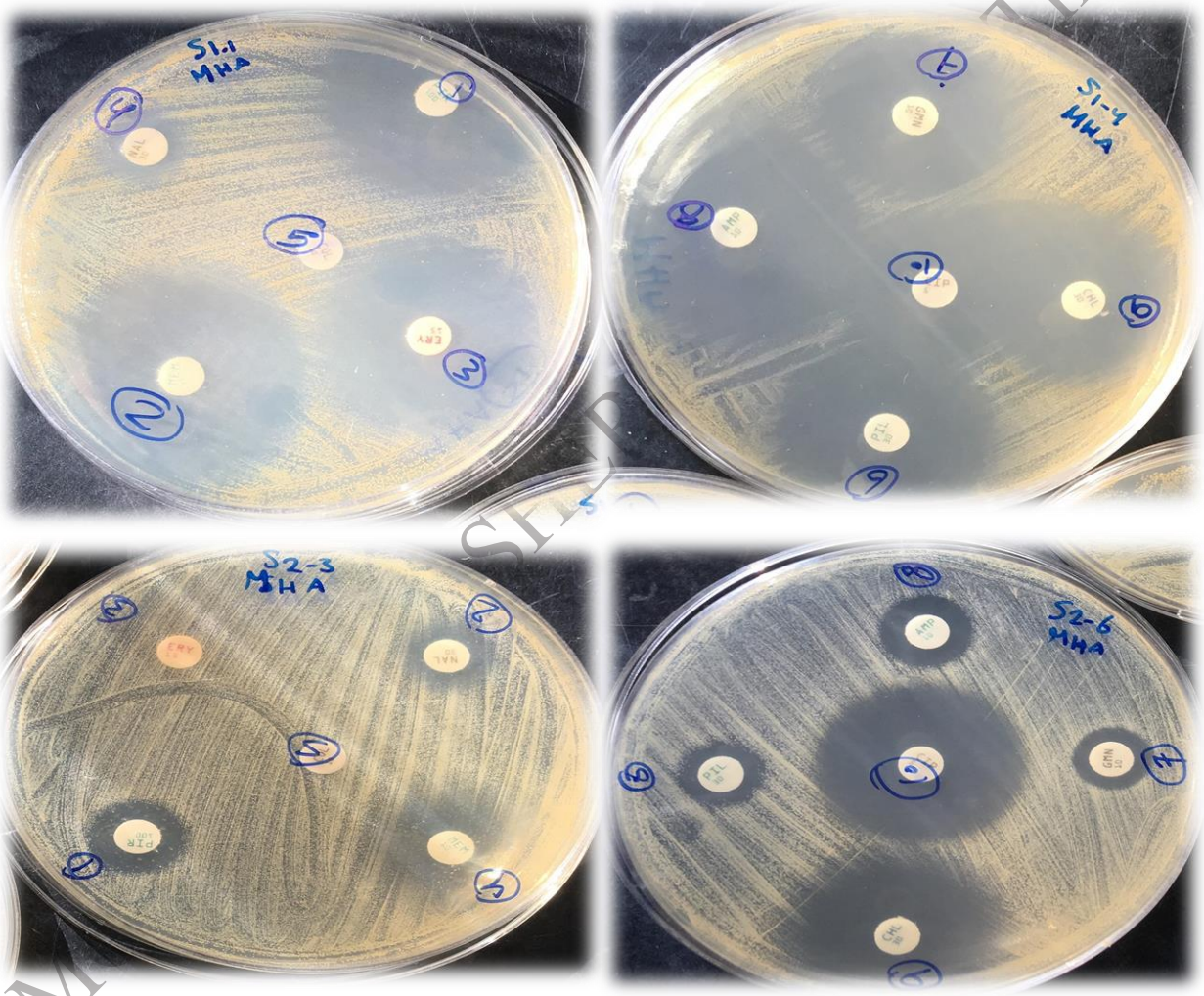


Figure 4. Growth of *S.aureus* B.01755 (marked as S1) and B.02174 (marked as S2) strains in the presence of the tested ten different antibiotics.

Table 3. Antibiotic susceptibility for *S. aureus* B.01755 and *S. aureus* B.02174

No.	Tested antibiotic	*S. aureus B.01755*		*S. aureus B.02174*		Reference		
		mean (mm)	indication	mean (mm)	indication	resistant	intermediate	susceptible
1	Piperacillin 100µg	34.3	susceptible	11.7	resistant	---	---	>18-21
2	Nalidixic Acid 30µg	10.0	resistant	9.7	resistant		---	>19
3	Erythromycin 15µg	30.0	susceptible	0.0	resistant	≤13 14	22	≥23
4	Meropenem 10µg	35.3	susceptible	12.0	resistant	≤13 14	15	≥16
5	Colistin 10µg	0.0	resistant	0.0	resistant	≤8	9 – 10	≥11
6	Piperacillin 30µg	30.3	susceptible	10.3	resistant	---	---	>18-21
7	Gentamycin 10µg	25.7	susceptible	11.0	resistant	≤12	13 – 14	≥15
8	Ampicillin 10µg	36.0	susceptible	13.7	resistant	≤28	---	≥29
9	Chloramphenicol 30µg	27.7	susceptible	27.0	susceptible	≤12	13 – 17	≥18
10	Ciprofloxacin 5µg	24.0	susceptible	29.0	susceptible	≤15	16 – 20	≥21

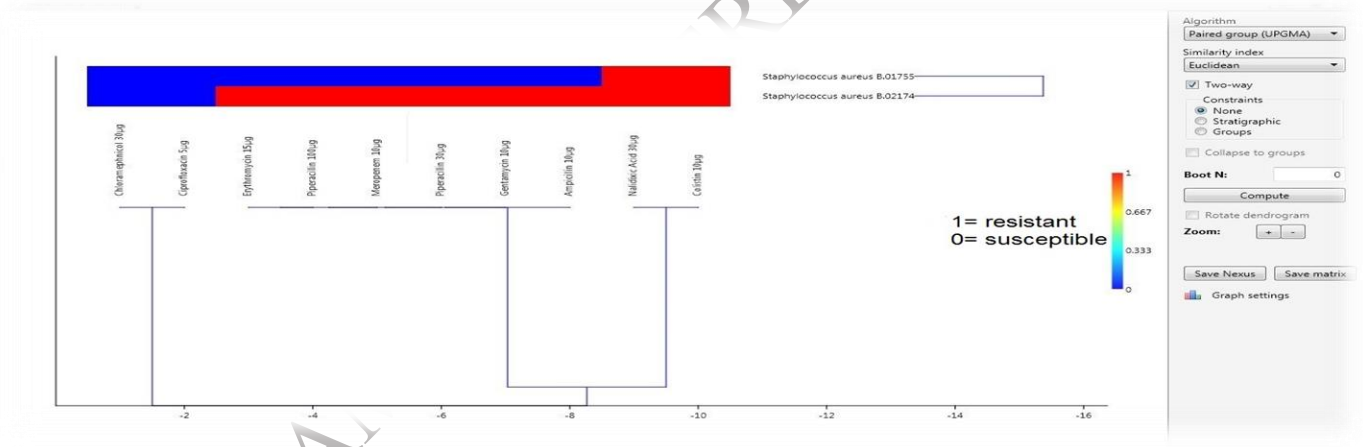


Figure 5. Similarity tree of *S. aureus* strains based on their susceptibility to the tested antibiotics.

Despite a common ground between the two strains, in general, strain B.01755 was more susceptible towards antibiotics than B.02174 (Figure 4, Table 3). While B.01755 was only resistant to Nalidixic Acid 30 µg and Colistin 10 µg, B.02174 was resistant to all the 10 antibiotics tested except Chloramphenicol 30 µg and Ciprofloxacin 5 µg, confirming this strain as MRSA. Both strains showed resistance to Nalicidic acid, which is the firstly used quinolone. It targets bacterium DNA either through gyrase enzyme or topoisomerase IV. Bacteria has developed resistant to it by horizontal gene transfer that results in alteration in the antibiotic targets configuration (Pham et al., 2019). The drug is more suitable for treating Gram-negative bacteria living in the human urinary tract (Miller, 1979.) than Gram-positive microorganisms. Similarly, colistin is found to be almost exclusively effective against Gram-negative bacteria rather than Gram-positive (Gurjar, 2015). It was mostly used as a therapy against Enterobacteriaceae (Kempf et al., 2016). Beta-lactam containing antibiotics, such as Piperacillin (100 µg and 30 µg) that is easily degradable by beta-lactamase enzyme, and Meropenem 10 µg were able to inhibit the growth of B.01755, thus proved to be effective antibiotics against this strain (susceptibility level was determined by clearing zones of 34.3 mm, 30.3 mm, and 35.3 mm, respectively). This observation also suggests the absence of beta-lactamase enzyme and Penicillin Binding Protein PBP2a from that strain. In contrast, B.02174 showed a strong resistance (means of clearing zones were 11.7 mm, 10.3 mm, and 12 mm, respectively) towards the beta-Lactam-containing antibiotics applied in the study. Different phenomena can be assumed behind this observation: either this strain is able to produce beta-lactamase enzyme, or it possesses a PBP2a protein (encoded by *mecA* gene) in its cell wall. It was reported (Lowy, 2003) that the plasmid which holds resistance to penicillin in *S. aureus* is a large plasmid that also codes for resistance to other antibiotics, including gentamicin and erythromycin. This can illustrate the reason behind *S.aureus* B.02174 to be resistant for beta-lactam antibiotics (piperacillin, ampicillin, meropenem) along with other antibiotics such as erythromycin and gentamycin. In contrast, B.01755 strain showed susceptibility to all of beta-lactams, gentamicin, and erythromycin. This observation might suggest that *S.aureus* B.02174 hold resistance genes for beta-lactams as well as other antibiotics in mobile genetic elements that are absent from the susceptible strain. Both bacterial strains were susceptible to chloramephnicol, in agreement with other studies (e.g. Fayyaz et al., 2012) where majority of MRSA strains isolated from medical specimen (75.8%) were susceptible to that antimicrobial. Ciprofloxacin is a drug

from fluoroquinolone family, it targets topoisomerase IV, and DNA gyrase in *S. aureus* (Campion et al., 2004). The state of susceptiblity for both bacterium to ciprofloxacin suggests that there is no genetic elements encding for resistance in both of them.

To visualize better the difference between the antibiotic susceptibility of the two *S. aureus* strains (Figure 5), a similarity tree was constructed using PAST 4 software (Hammer et al., 2001) Both strains showed similarity in the case of four antibiotics; susceptible for chloramphenicol 30 µg and ciprofloxacin 5 µg, and resistant for colistin 10 µg and nalidixic acid 30 µg. In the cases of the remaining 6 antibiotics the resistance patterns were diverse.

6.2.Effects of stress factors on *S. aureus* B.02174 resistant strain

S. aureus B.02174 has been exposed to different treatments (0% NaCl and no irradiation as a control sample, 6% NaCl, 0.6 kGy gamma irradiation, and coupled treatment of 6% NaCl with 0.6 kGy gamma irradiation). Inoculation on TSA (for determination of surviving cell concentration) and MHA (for antibiotic susceptibility testing) have been performed. In the case of antibiotic susceptibility test the previously applied 10 antibiotics were placed onto the MHA. Findings of 24-hours incubation revealed that viable (surviving) counts decreased in the cases of all treatments compared to control sample (Figure 6).

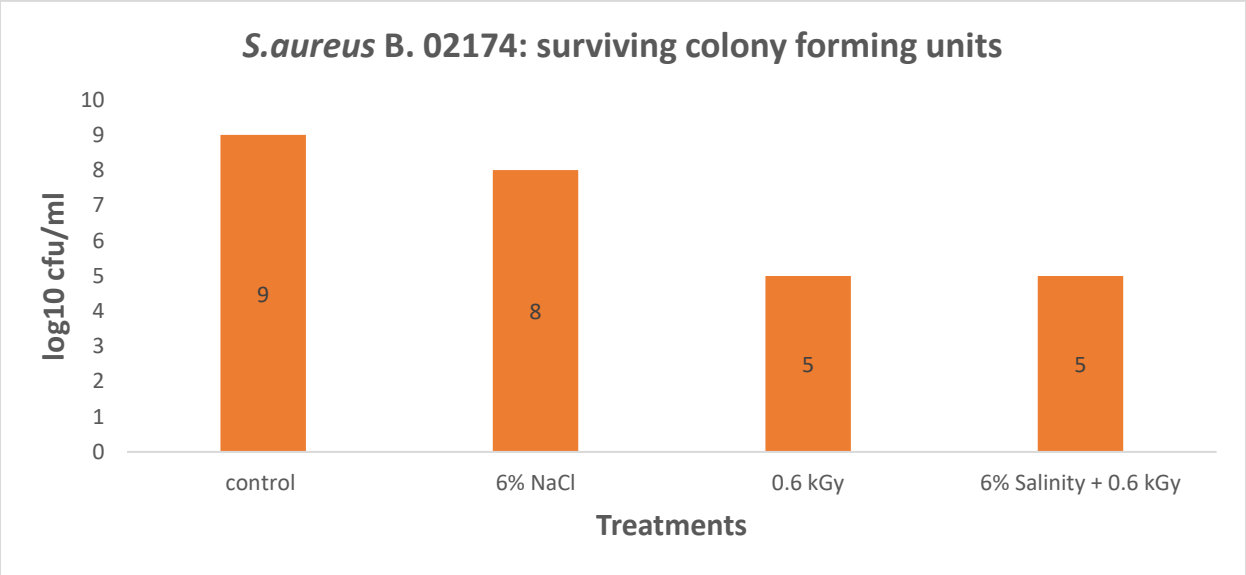
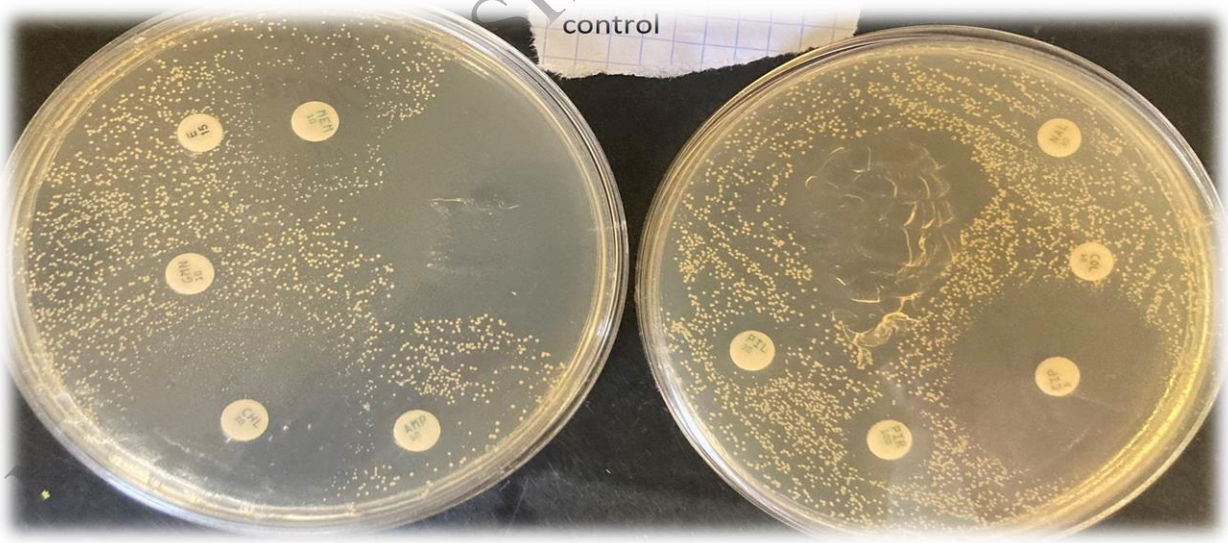


Figure 6. Log_{10} colony forming units counted on TSA for *S. aureus* B.02174 after treatments

Only one log decline in the bacterial count was detected in the presence of 6% NaCl, agreeing with other data showing minimal effect on cells shape and numbers up to 10% NaCl (Feng et al., 2022).

0.6 kGy irradiation alone or combined with 6% salt had more significant effect by decreasing the number of surviving cells with 4 logs, indicating the highest effect of irradiation. This low dose of gamma irradiation used in our study is suggested to be as efficient in decreasing the total viable cells count as the dose recommended by the Food and Drug Administration of the United States (5 logs decrease) (Linton, 2001). As noted in the literature review, the effect of gamma irradiation on bacterial number varies depending on many factors, including (but not exclusive to) the strain, the substrate or food irradiated, environmental factors as well as the irradiation dose and time of exposure. In the study of Farkas & Mohácsi-Farkas (2011), doses from 0.26 kGy to 0.57 kGy were considered as D_{10}-values in non-frozen meat for *S. aureus.* Our treatment was done in PBS solution, which might offer more free water molecules than meat matrix, allowing generation for more free radicals. This might explain the relatively high decrease in bacterial numbers in our study, and gave 0.15 kGy as D_{10} value. Van Gerwen et al. (1999) stated in their review that various researchers had reported inconsistent and even conflicting results in terms of the measured D_{10} values for gamma irradiation of identical objects.

Antibiotic resistance showed different responses regarding the treatments and depending on the type of antibiotics (Figure 7, Table 4).



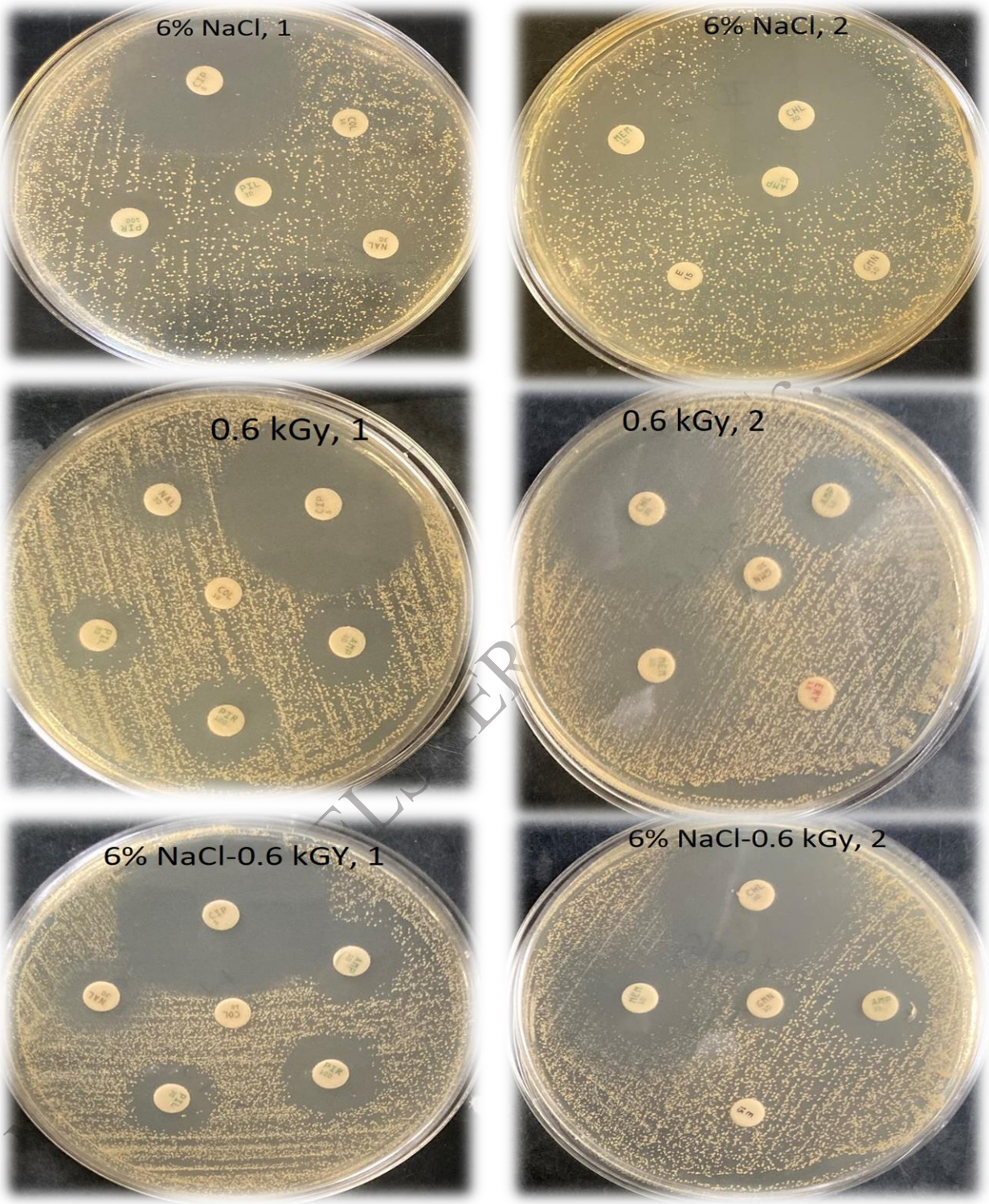


Figure 7. Results of disk diffusion test on MHA medium for *S. aureus* B.02174 after the indicated treatments

Table 4. Antibiotic susceptibility for *S. aureus* B.02174 after treatments by 6% NaCl, 0.6 kGy, and combined 6% NaCl with 0.6 kGy gamma irradiation.

Tested Antibiotic		control		6% NaCl		0.6 kGy		6%NaCl+0.6 kGy	
		mean (mm)	indication	mean (mm)	indication	mean (mm)	indication	mean (mm)	indication
1	Piperacillin 100	12.7	resistant	13.0	resistant	16.5	resistant	15.8	resistant
2	Nalidixic Acid 30µg	9.7	resistant	10.0	resistant	10.3	resistant	12.8	resistant
3	Erythromycin 15µg	0.0	resistant	0.0	resistant	0.0	resistant	0.0	resistant
4	Meropenem 10µg	14.0	resistant	15.0	intermediate	16.3	susceptible	16.3	susceptible
5	Colistin 10µg	0.0	resistant	0.0	resistant	0.0	resistant	0.0	resistant
6	Piperacillin 30µg	10.7	resistant	11.0	resistant	14.3	resistant	13.8	resistant
7	Gentamycin 10µg	8.3	resistant	9.3	resistant	10.3	resistant	10.3	resistant
8	Ampicillin 10µg	11.0	resistant	13.0	resistant	15.5	resistant	15.3	resistant
9	Chloramphenicol 30µg	30.0	susceptible	31.0	susceptible	32.3	susceptible	33.5	susceptible
10	Ciprofloxacin 5µg	29.7	susceptible	33.8	susceptible	35.0	susceptible	36.5	susceptible

Tested Antibiotic	Reference for susceptibility		
----------	Resistant	Intermediate	Susceptible
Piperacillin 100	---	---	>18-21
Nalidixic Acid 30µg	---	---	>19
Erythromycin 15µg	≤13 14	22	≥23
Meropenem 10µg	≤13 14	15	≥16
Colistin 10µg	≤8	9 – 10	≥11
Piperacillin 30µg	---	---	>19
Gentamycin 10µg	≤12	13 – 14	≥15
Ampicillin 10µg	≤28	---	≥29
Chloramphenicol 30µg	≤12	13 – 17	≥18
Ciprofloxacin 5µg	≤15	16 – 20	≥21

As shown in (Figure 8, Table 4), the response of *S. aureus* B.02174 in the case of Erythromycin 15 µg and Colistin 10 µg remained unchanged (as resistant), exhibiting zero clearing zones around the antibiotic disks, suggesting no change in their susceptibility pattern by the treatments. The bacterium was susceptible to Chloramphenicol 30 µg and Ciprofloxacin 5 µg before treatments, and these susceptibilities increased by stressing the cells with 6% salt and 0.6 kGy, showing the highest effect in the case of combined treatment (6% NaCl and 0.6 kGy irradiation). Resistance for Ampicillin 10 µg, Piperacillin 30 µg and 100 µg, and Gentamycin 10 µg did not change after the treatments, this might indicate that neither high salinity nor irradiation caused any change in the genes responsible for resistance or in their expression. However, effect of 0.6 kGy gamma irradiation and combination of salt and radiation increased the sizes of the clearing zones to a better extent than 6% NaCl alone. The bacterium displayed resistance for Meropenem 10 µg in the control sample, at the same time this resistance changed to intermediate level due to the increased level of NaCl (up to 6%). Moreover, 0.6 kGy gamma irradiation alone or combined with 6% of salt could increase the size of the clearing zone to susceptible level (Figure 8).

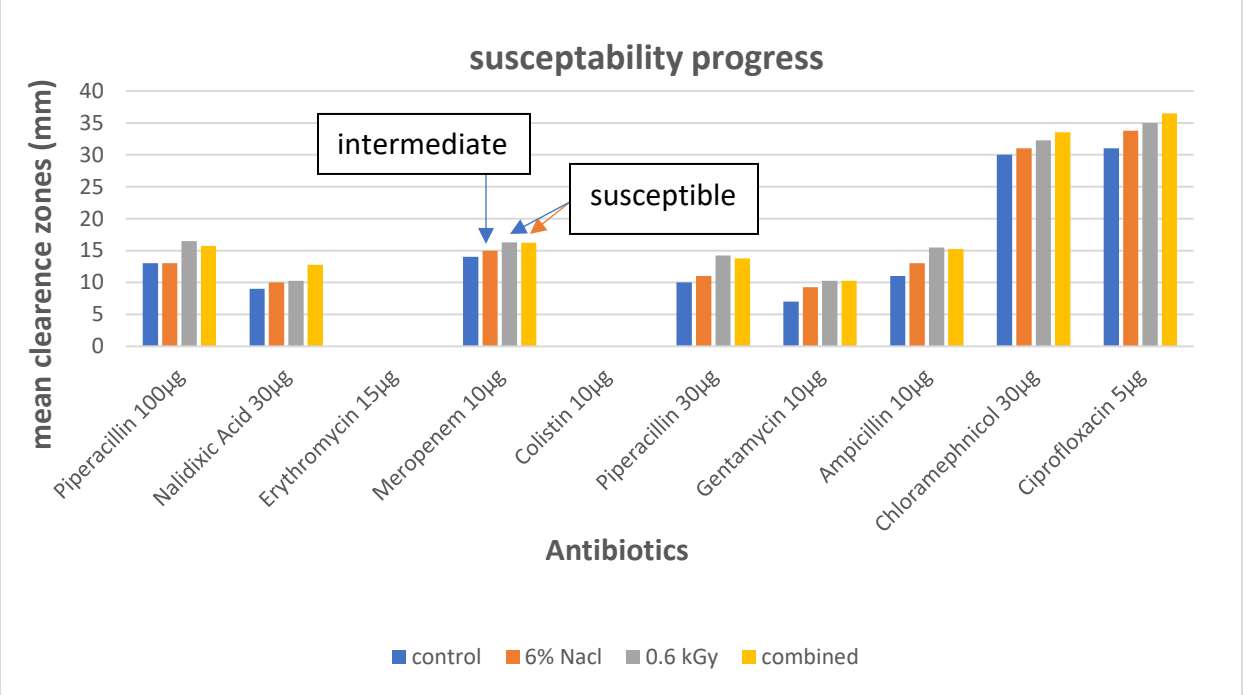


Figure 8. Changes in antibiotic sensitivity/resistance pattern for *S. aureus* B.02174 after treating the cells by sublethal level of gamma radiation, 6% NaCl, or their combination after treatments.

Meropenem is an antibiotic belonging to the carbapenem family of antimicrobials. It is a stable penicillin against most of penicillinases, therefore it has ultra-broad spectra of antibacterial activity, treating many Gram-negative and Gram-positive bacteria (Drusano, 1997). It can bind to PBP2 proteins in Gram-positive and Gram-negative bacteria, leading to inhibition of their cell wall synthesis and growth. Meropenem has several additional structural features that give it advantages over other carbapenems such as imipenem, particularly its stability to dehydropeptidase-l metabolism, a human renal enzyme degrading imipenem. The structure of meropenem contain a five-membered thiazolidine ring that differs from that of penicillin's in several ways. Instead of a sulfur atom at position 1, there is a carbon atom, and there is an unsaturated bond between the carbon atoms at position 2. These alterations allow it to escape the degradation by beta-lactamases. Meropenem is a beta-lactam that kill bacteria by binding to penicillin-binding proteins (PBPs), thereby inhibiting peptidoglycan responsible for cell wall biosynthesis. Meropenem can bind to all four PBPs identified in *S. aureus* (Drusano, 1997).

Similarity tree, (Figure 9). was obtained using Past 4-12 (Hammer et al., 2001) to visualize the changes in *S.aureus* response after treatments.

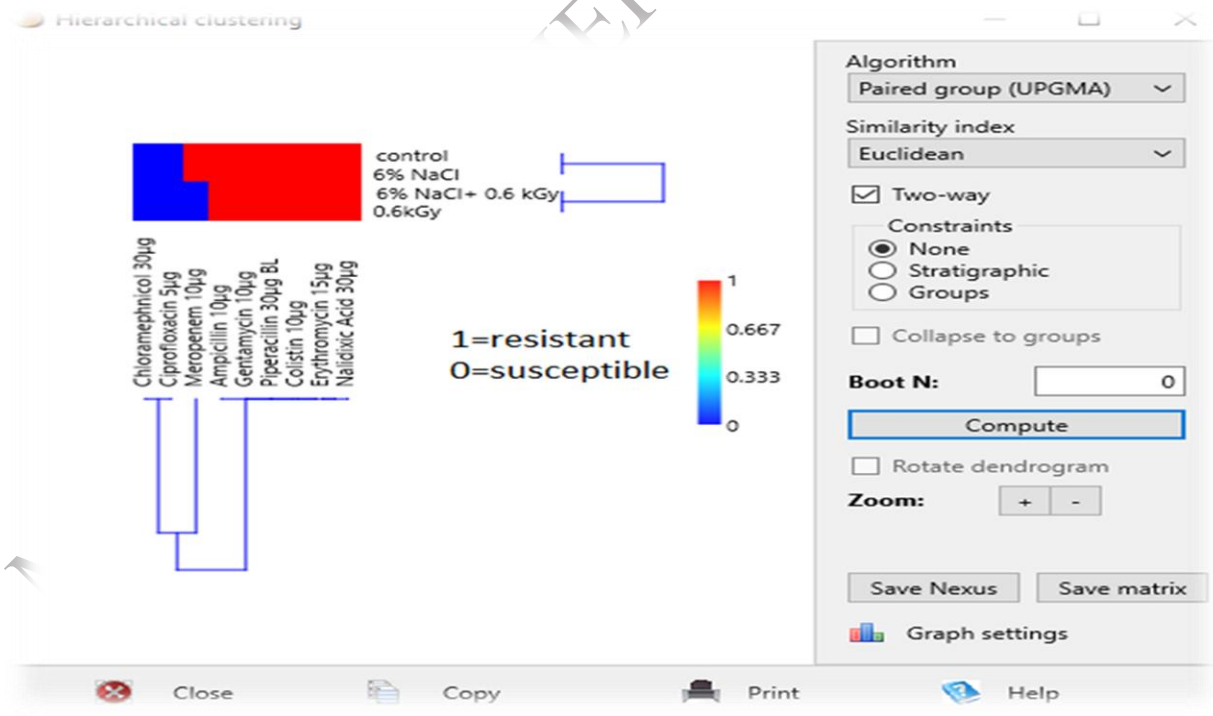


Figure 9. Similarity tree for susceptibility/resistance for ten antibiotics after treatment by 6% NaCl alone and in combination with 0.6 kGy radiation.

6.3.Effect of increased level of NaCl

The increasement of salt concentration by adding 6% NaCl to PSB resulted in a shift in bacterium response to meropenem: the resistance turned to intermediate susceptibility. By increasing the concentration of NaCl up to 10 and 12% with or without irradiation, bacterial susceptibility to meropenem was further improved (table 5). This increase led to shifting the bacterial antibiotic susceptibility from resistant to intermediate. Moreover, there was a decline in the bacterial sensitivity against meropenem with increasing salt concentration, suggesting that higher salt concentrations initiated some bacterial self defense mechanisms, or that high NaCl decreases the binding efficacy of antibiotic to PBPs. Response to irradiation as standalone treatment or combined with 6% salt concentration increased susceptibility towards meropenem. However, the higher salt concentrations (10 and 12%) combined with 0.6 kGy gamma irradiation were responsible for bigger clearing zones around the antibiotic disks, that exceeded the susceptibility level. There was almost no growth on MHA after treatments with 10 and 12% salt and irradiation combination, therefore, Petri dishes were incubated for further 72 hours at 37 °C to be able to measure the sizes of clearing zones (Figure 10).

Table 5. Changes of antibiotic susceptibility for *S. aureus* B.02174 after 6%, 10%, and 12% NaCl treatment, irradiation with 0.6 kGy, and combination of NaCl treatments with 0.6 kGy gamma radiation.

	Treatment	Mean clearing zones for meropenem (mm)	Susceptibility
1	**control**	14.0	**resistant**
2	**6% NaCl**	16.0	**intermediate**
3	**10% NaCl**	15.7	**intermediate**
4	**12% NaCl**	15.3	**intermediate**
5	**control+0.6% kGy**	17.7	**susceptible**
6	**6% NaCl+0.6% kGy**	18.3	**susceptible**
7	**10% NaCl+0.6% kGy**	(28.0)	**susceptible**
8	**12% NaCl+0.6% kGy**	(24.3)	**susceptible**

Results were statistically significant as ($P<0.05$) (Tables 6-7), and the tests were repeated twice to confirm the results, and the results obtained were similar. These finding may be elucidated that increased stress by salinity in combination with irradiation led to a direct damage to cell wall structure that allowed the antibiotic to diffuse more easily in the cells and cause inhibition, especially with the higher salt concentrations of 10 and 12 %, respectively. Another justification

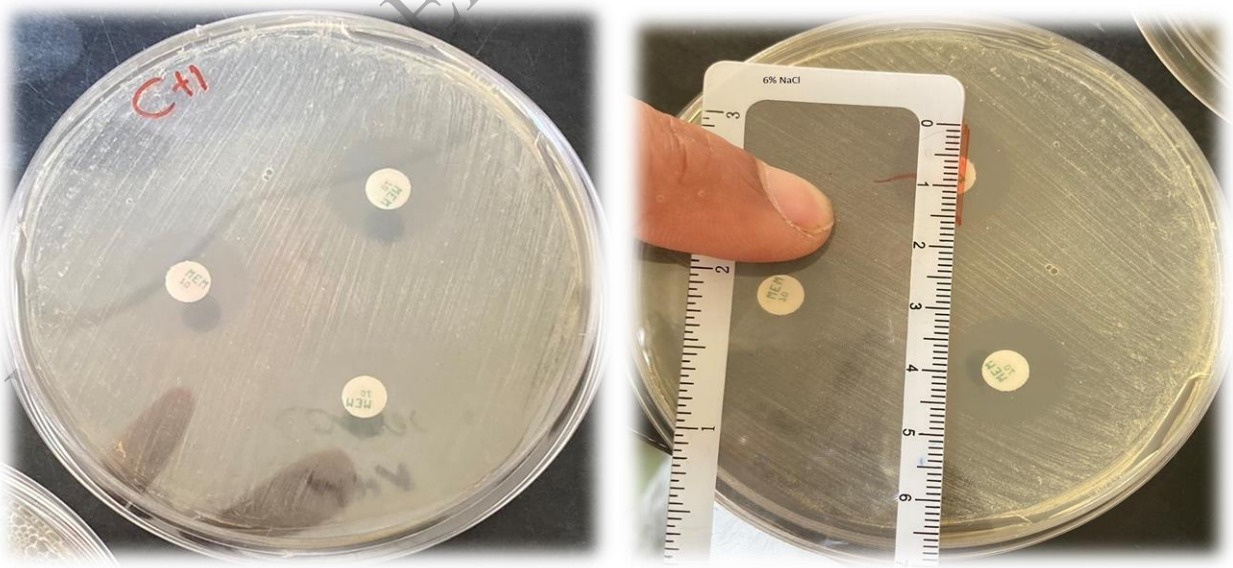
could be a damage in the bacterial nucleic acids, which was caused by the harsh environmental stress (saline-irradiation). Therefore, further investigation for the detection of *mecA* gene was done.

Table 6. Single Factor ANOVA summary, it shows the means between repeated samples and variances.

Groups	*Count*	*Sum*	*Average*	*Variance*
control	3	42	14	0
6% Nacl	3	48	16	1
10% Nacl	3	47	15.66667	0.333333
12% Nacl	3	46	15.33333	0.333333
control+0.6% KGy	3	53	17.66667	0.333333
6% Nacl+0.6% KGy	3	55	18.33333	0.333333
10% Nacl+0.6% KGy	3	84	28	12
12% Nacl+0.6% KGy	3	73	24.33333	0.333333

Table 7. ANOVA test. It shows the P value to be less than 0.05.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	508	7	72.57143	39.58442	6.19E-09	2.657197
Within Groups	29.33333	16	1.833333			
Total	537.3333	23				



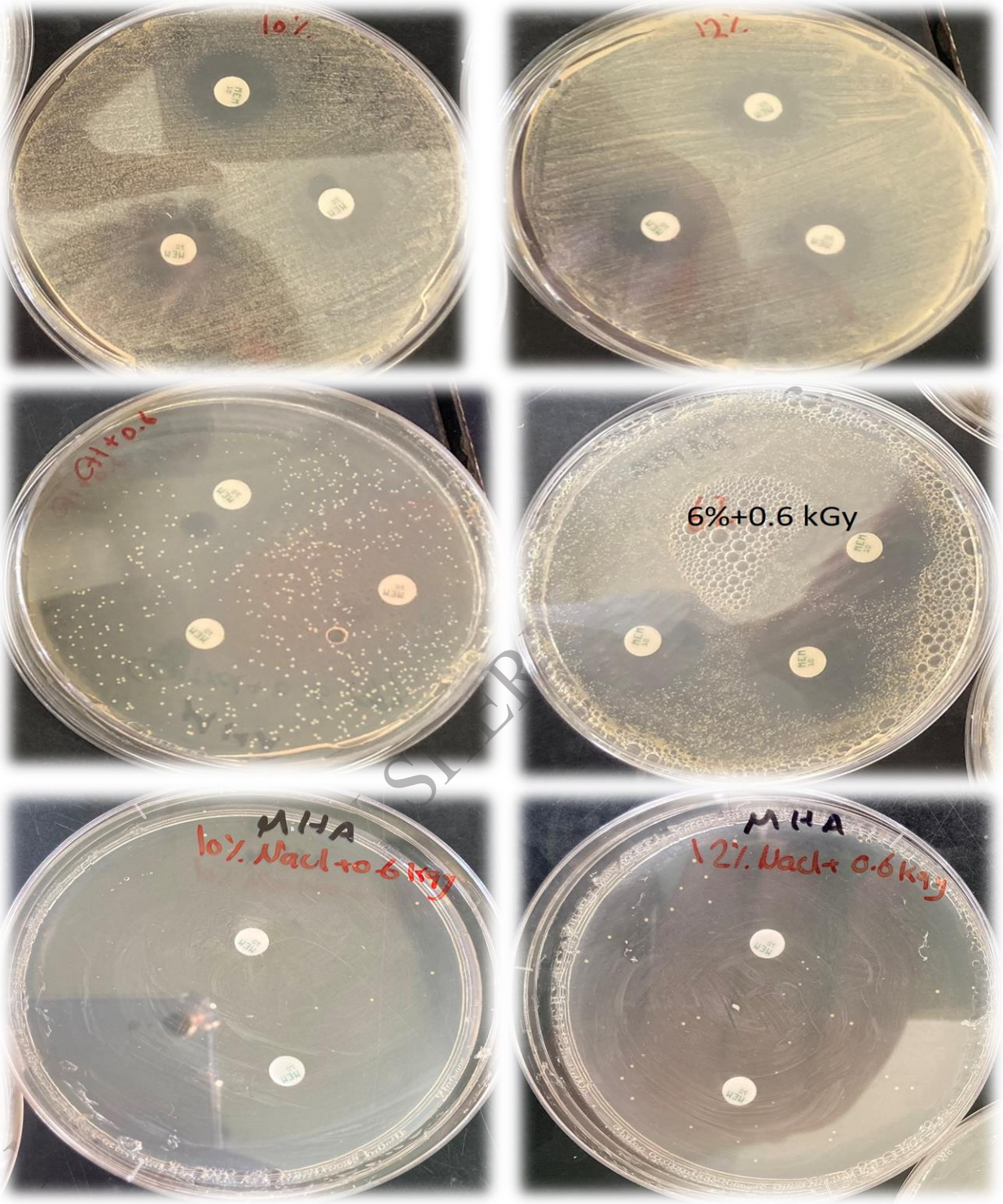


Figure 10. Results of antibiotic disk diffusion agar test on MHA medium for *S. aureus* B.02174 after treatments with 6, 10, 12% NaCl alone, or in combination with 0.6 kGy gamma irradiation.

Numbers of surviving cells revealed the same ratio in decreasing bacterial cell counts due to salt addition with or without gamma irradiation. The applied NaCl concentrations (6,10, and 12%) caused a decline in the bacterial count by one log unit, from 10^8 CFU/ml to 10^7 CFU/ml.
Four-log-unit decrease was observed as result of 0.6 kGy radiation, and the same 4-log-unit decline has been achieved by applying 0.6 kGy dose with higher NaCl concentrations, as it is shown in Figure 11.

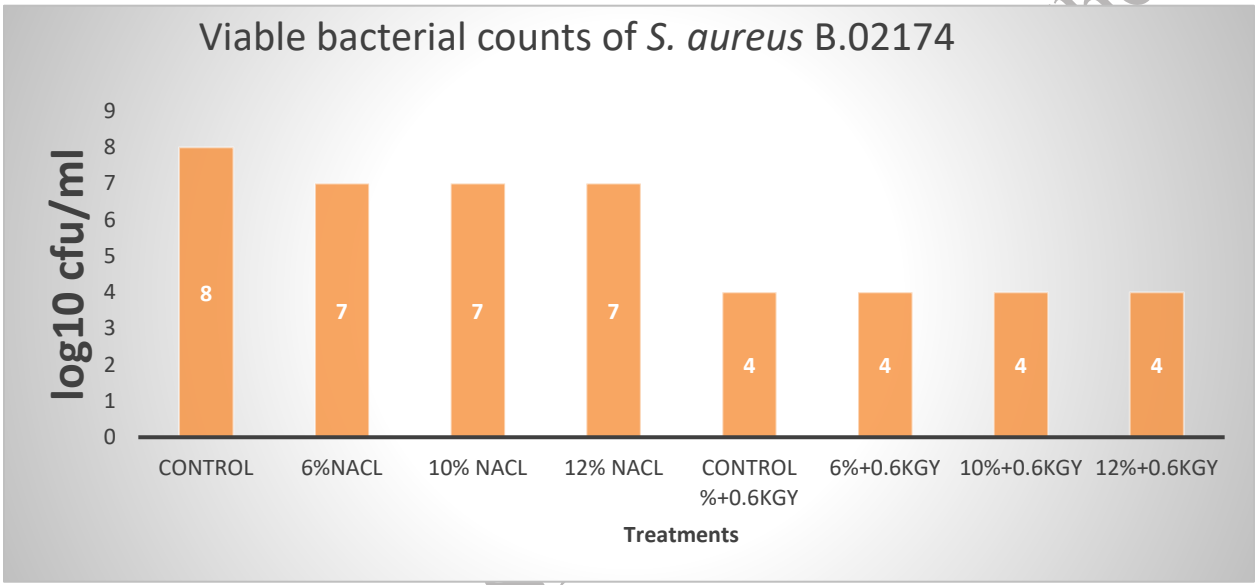


Figure 11. Viable bacterial counts on TSA medium for *S. aureus* B.02174 after treatments with 6%, 10%, and 12% NaCl alone, or combined with 0.6 kGy gamma irradiation.

The findings revealed that the sensitivity of *S. aureus* B.02174 to meropenem antibiotic shifted from resistant to intermediate when the bacterial suspension was supplemented with up to 12% salt. Furthermore, exposure to gamma irradiation, with or without salt addition resulted in susceptibility to the antibiotic and significant decrease in viable cell number.

6.4.PCR test

In order to explore the possibilities behind the change in meropenem resistance, presence of *mecA* gene encoding for penicillin binding protein 2a (PBP2a) (and among others, it can be responsible for β-lactam resistance of *S. aureus*) was detected by PCR method. The traditional PCR test targeted a 125-base pair region of the *mecA* gene. Both intracellular DNA (iDNA) and extracellular DNA (eDNA) were analyzed to assess the bacterium's response to salt, irradiation, and salt-

irradiation treatments. Amplification of the 125-base pair long DNA of the *mecA* gene confirmed its presence in the cases of almost all samples, as depicted in Figure 12. From the results it can be seen that despite treatment by gamma irradiation the *mecA* gene remained amplifiable from the eDNA and iDNA fractions of treated *S. aureus* resistant cells, respectively. As gamma radiation usually causes single or double stranded DNA break, sublethal dose (0.6 kGy) of Co^{60} was not sufficient enough for significant amplifiable DNA decrease, and the presence of the gene remained detectable. However, influence of NaCl on the PCR efficiency can also be seen from Figure 12, as presence of 12% salt in the eDNA sample influenced the amplicon generation negatively.

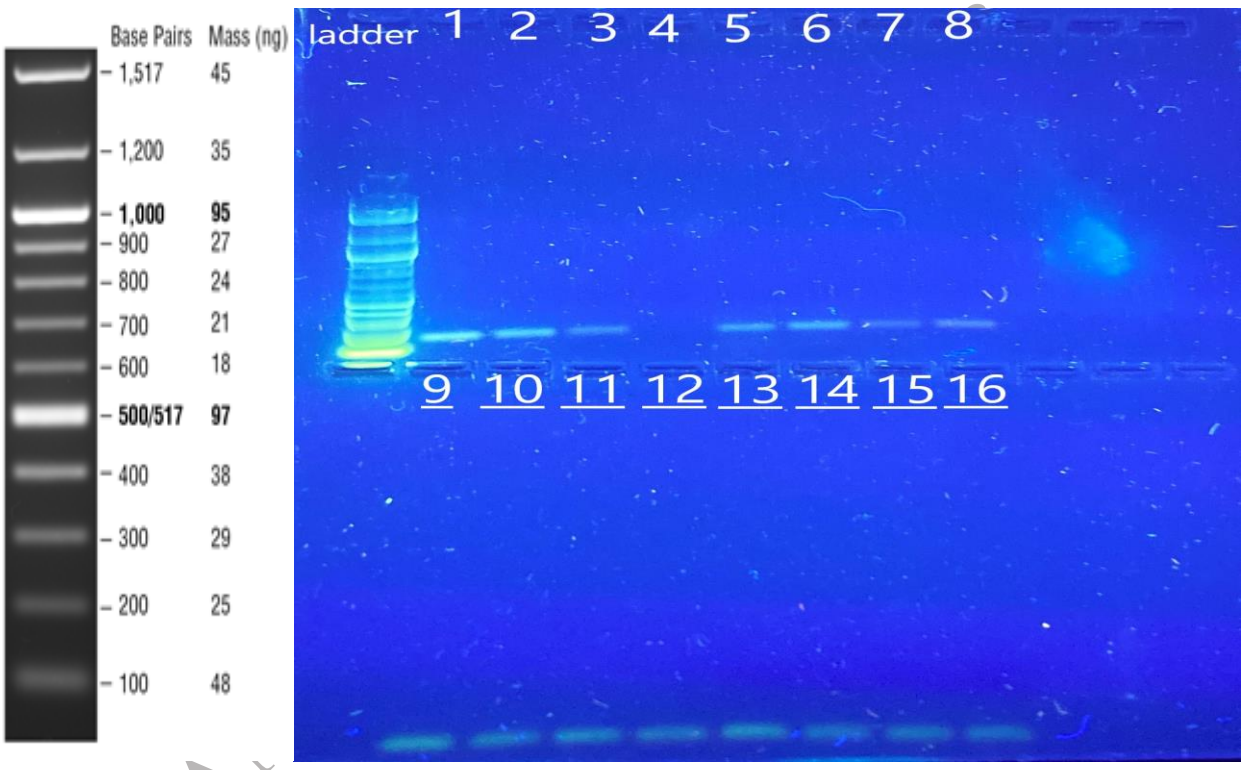


Figure 12. PCR amplicons of *mecA* gene detected on 1% agarose gel using horizontal gelelectrophoresis. Samples from 1 to 8 represent eDNA and from 9 to 16 represent iDNA. (1,9) control – (2,10) 6% NaCl- (3,11) 10 % NaCl- (4,12) 12 % NaCl- (5,13) 0% NaCl irradiated by 0.6 kGy- (6,14) 6% NaCl irradiated by 0.6 kGy - (7,15) 10% NaCl irradiated by 0.6 kGy -(8,16) 12% NaCl irradiated by 0.6 kGy.

However, it should be noted that the presence of the *mecA* gene does not necessarily mean that its expression is unchanged. As a result of stressors, changes in the expression of the target gene can

occur, and although the presence of *mecA* can be detected by PCR, the completion of transcription could only be examined by reverse transcription (RT) PCR analyzing the presence of mRNA molecules.

Moreover, these results suggest that the treatments did not affect the bacterium's genetic response, indicating that changes in antibiotic susceptibility were likely due to increased permeability to antibiotics under high salt concentrations and damage to the cell walls, which may have enhanced cells' absorption of antimicrobial compounds.

7. Conclusions

In this study altered behaviour of *S. aureus* B.02174 strain was determined in the case of meropenem, a β-lactam antibiotic as a result of gamma irradiation and salinity stress. (Madiraju et al., 1987) observed that NaCl enhances the resistance for methicillin in the case of *S. aureus* by stimulating production of penicillin binding protein 2' and providing osmotic support. It was observed in this study that *mecA* gene from iDNA fraction of B.02174 strain was amplifiable even in presence of 12% NaCl, thus loss of resistance can be explained by either the inhibition of gene expression, or the altered permeability of the membrane. NaCl at the relatively high concentration (>0.3 M) could enhance the porin permeability and/or damage bacterial membranes, increase cell membrane permeability, which consequently enhances bacterial uptake of diverse molecules from the outer environment (Chen et al., 2022). In a previous study by Kovács and co-workers (unpublished results) it was shown that 0.6 kGy gamma irradiation can reduce the quantity of amplifiable *mecA*, however this dose proved to be insufficient for total inactivation of this gene. Based on the observations of this study it can be hypothesized that irradiation had an indirect effect on *S. aureus* DNA (by forming free radicals) and accumulated effect of the two applied stressors contributed to the change of resistance into sensitivity in the case of meropenem, a carbapenem antibiotic.

8. Summary

S. aureus is a commensal-opportunistic bacterium that is becoming more dangerous and imposes a considerable health risk to humans. It belongs to Gram-positive bacteria, and although the microbe is easily degraded by cooking or pasteurization (heat treatments), it is capable of secreting very heat-stable toxins if foods are cross-contaminated. Staphylococcal food intoxications are one of the significant health risks endangering humans from a food safety perspective. Therefore, food handlers must be in a responsible position to ensure the safety of foods and not allow cross-contamination. *S. aureus*'s ability to evolve is very high. It is well known for adapting stress factors such as high salinity and is resistant to most antibiotics used for medication. The rates of reported resistant strains of *S. aureus* have arisen since the first antibiotic's discovery, which led to untreatable infections from mild skin rashes to severe sepsis symptoms, and even death. The scientific community has been in a war against superbugs like MRSA. Methicillin-resistant staphylococcus is challenging to treat due to its adaptation and acquisition of resistance genes.

Along with microorganisms' development, humankind has been developing new technologies for food preservation. Traditional food preservation techniques included heat treatment and non-heat treatments such as salting. As *S. aureus* accompanies humans everywhere, they live on their skins and nasal tracts, its presence is likely and has been reported in highly salted foods such as semi-solid cheeses. Despite the high osmosis of such foods, *S. aureus* remains able to grow and secret its toxins. Therefore, it is suitable to examine to what extent of salinity affects its growth and relate it with its ability to resist antibiotics, the current defense line for humans against microbes.

Sophisticated and newly introduced food preservation techniques include food irradiation. It is a promising technology that many research efforts showed its minimal effect on foods' nutritional and organoleptic characteristics while having a hugely damaging effect on undesired organisms such as pests, parasites, and microorganisms. Food irradiation is becoming more accepted with consumers becoming more open to minimally processed foods, particularly fresh produce.

In this study two strains were selected for examination; their susceptibility was compared in the presence of 10 various antibiotics. The most resistant strain was further challenged with three different table salt concentrations 6 %, 10 %, and 12%. Irradiation with a sublethal dose of 0.6 kGy gamma irradiation was introduced, alone or combined with NaCl treatment. The results showed that treatments of sublethal dose of gamma irradiation alone or combined with high osmosis for the MRSA strain studied increased its susceptibility against meropenem, a beta-lactam

antibiotic. However, this was not reflected in the genetic profile of the bacterium. *mecA* gene which is responsible for MRSA resistance against beta-lactam drugs was present for almost all the treatments except for one sample that was high in salt (12%), which probably affected the efficacy of PCR testing. The finding is important as an indicator that high saline foods can increase *S. aures* sensitivity against meropenem (and even to additional antibiotics), and even low doses of gamma irradiation are beneficial in decreasing the living population of *S. aureus*. Its effect in decreasing the microbial resistance against meropenem, which is an antibiotic belonging to carbapenem group of antimicrobials and widely used antibiotic at present is significant.

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