Potential Inhibition of Lactate Dehydrogenase by Oxamate in *Enterococcus faecalis*

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JULY, 2021

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Copyright Statement

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Abstract

E. faecalis is an ancient bacteria responsible for a lot of nosocomial infections in human beings. One of the main reasons for its survival over millions of years is its resistance to various physiological stresses and also to antibiotic drugs used for its treatment. The acquired antibiotic resistance in the bacteria is major concern of the modern medicine. *E. faecalis* depends majorly on the enzyme Lactate Dehydrogenase (LDH) which maintains redox balance for growth, resistance and its virulence. Oxamate, the salt of oxamic acid, is a compound with a similar structure to pyruvate and is used as an anticancer agent worldwide that can competitively bind to LDH and inhibit its activity. Further evidence of oxamate's binding with LDH is also provided by computational approaches. In this study we attempted to evaluate the antibacterial effect of oxamate on *E. faecalis* by inhibiting LDH enzyme. We found out the minimum inhibitory concentration of oxamate after adding its different concentrations (ug/mL) to the bacterial culture and running it on the 96-well plate on the microplate reader. Maximum growth inhibition was shown in higher doses of inhibitor (100, 150, 200 ug/mL) while little to no inhibition was shown on smaller doses (5, 10, 25, 50, 75 ug/mL). We employed six different stressors, SDS, H_2O_2, Ethanol, DMSO, Glucose and HOCl, in addition to the minimum inhibitory concentration of oxamate. A moderate level of inhibition was shown in the the cultures containing MIC of oxamate with SDS and Glucose, while little to no inhibition was shown in the cultures contaning oxamate with DMSO and H_2O_2. A significant amount of inhibition was shown in the culture containing the inhibitor with Ethanol. With everything into account, it was seen that activity of LDH was inhibited strikingly by oxamate.

Key words: *E. faecalis,* Lactate dehydrogenase (LDH), Oxamate

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CHAPTER#1: INTRODUCTION

1.1 *Enterococcus faecalis:* A Pathogen

Enterococci are ancient bacteria that are thought to have existed at least since the early Devonian period, or 412 million years ago, when mammals, reptiles, birds, and insects had their last common ancestor. Because of animal hosts' shedding, these bacteria are easily accessible in the environment and flourish in the oxygen-depleted and nutrient-rich environment of the intestines(Van Tyne et al., 2013).

E. faecalis	
Scientific name	*Enterococcus faecalis*
Domain	Bacteria
Family	Enterococcaceae
Order	Lactobacillales
Phylum	Bacitolla

Table 1 Taxonomic classification of *E. faecalis*

As typical commensals, enterococci live in the human gastrointestinal system, mouth cavity, and vagina. They can infect the biliary system, endocardium, abdomen, urinary tract, burn sites, and indwelling foreign devices, resulting in a wide range of illnesses in people. Endodontic infections have also been linked to enterococci (Halkai et al., 2016). *E. faecalis*, a gram-positive bacteria with a sphere-like form and diplococci-like arrangement, is a common microbe mostly found in animal and human guts and is known to cause a wide range of illnesses (Price et al., 2012). All the diseases and ailments caused by *E. faecalis* include bacteremia, endocarditis, pelvic, intra-abdominal, and soft tissue infections (Csonka & Hanson, 1991) and it is heavily concentrated when expelled into the environment through faeces. This bacterium is

regarded as a sign of feces-contaminated water containing dangerous microorganisms (Rince et al., 2000; Rincé et al., 2002).

1.1.2 *E. faecalis* and its Virulence

Enterococci possess traits that are commonly associated with pathogenesis, similar to other bacteria that cause diseases in humans. One of their inherent advantages is their ability to acquire, accumulate, and exchange extrachromosomal elements that carry virulence factors or genes that confer resistance to antibiotics. This adaptability helps them withstand harsh environmental conditions and partly accounts for their increasing significance as opportunistic pathogens. (Prestinaci et al., 2015). Out of the 14 or more species of enterococci, only *E. faecalis* and *E. faecium* are commonly found to colonize and cause infections in humans, with *E. faecalis* being isolated from 80% of human infections. (Huycke et al., 1998). Enterococci can cause infections like meningitis, hematogenous osteomyelitis, septic arthritis, and pneumonia, but these are less commonly observed. Pneumonia is a rare occurrence, mostly associated with ventilators and found in severely debilitated or immunocompromised patients who have received broad-spectrum antibiotics. There is no evidence suggesting that antibiotic-resistant enterococci, including VRE, are more or less likely to cause these infections compared to antibiotic-susceptible enterococci.

1.1.3 Treatment of *E. faecalis*

Antibiotics are medications used to treat infections caused by microorganisms, such as bacteria. These drugs are derived from bacteria and fungi that are not harmful to the patient being treated. Bactericidal antibiotics, which kill bacteria, are used to treat infections and conditions caused by *E. faecalis*, such as bacteremia, meningitis, and other hospital-acquired infections. These drugs are designed to target essential processes in the bacteria, making it easier to eliminate them (Rince et al., 2000). Some

of the commonly used antibiotics for treating bacterial infections are streptomycin, ampicillin, vancomycin and penicillin. They can be administered either as a single drug therapy for mild infections or in combination with other antibiotics for more severe infections.Bactericidal and Bacteriostatic drugs

Bactericidal drugs are used to treat bacterial infections and diseases such as bacteremia, meningitis, and other hospital-acquired infections. These medications are designed to target essential bacterial functions, making it easier to kill the bacteria. Individuals with weakened immunity against bacteria are advised to take these antibacterial drugs regularly. Examples of bactericidal drugs include beta-lactams also fluoroquinolones, and aminoglycosides. (Rince et al., 2000). Example of streptomycin that is a bactericidal is shown in Figure1

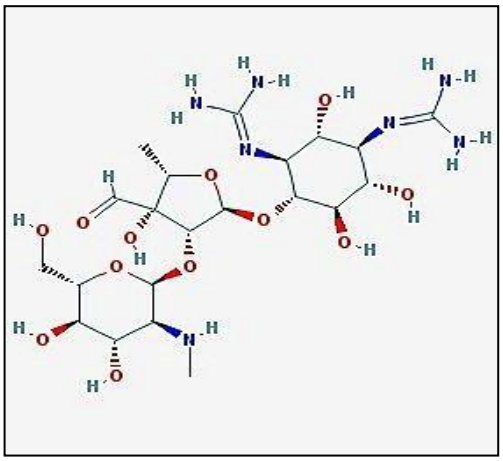


Figure 1 Streptomycin

Bacteriostatic drugs are helpful in preventing the growth of bacteria, which can lead to microbial cystitis. Some of the drugs that belong to this category are tetracycline, vancomycin, and penicillin. (Noskin et al., 1991) etc. Example of bacteriostatic drug vancomycin is shown in Figure1.2

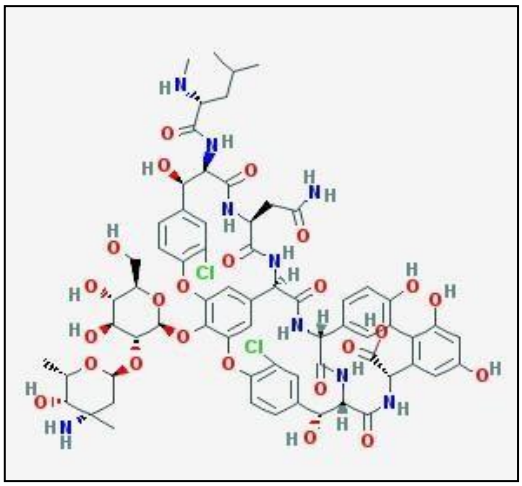


Figure 2 Vancomycin

1.1.3.1 Vancomycin

Vancomycin is a bacteriostatic drug (antibiotic) that was first discovered in 1953 from soil, and is produced by the organism *Amycolatopsis orientalis*. It is used to treat various gram-positive bacterial infections, such as skin infections, bloodstream infections, urinary tract infections, and endocarditis. Vancomycin works by preventing the formation of the cell wall by targeting and inhibiting the action of the building blocks. It binds to the amide bond of muramyl-pentapeptide at the terminal sequence, blocking the polymerase from extending the peptidoglycan backbone. As a result, the cross-link formations of growing chains by transpeptidase are inhibited. Enterococci developed resistance to vancomycin by acquiring genes through plasmids or transposons that allow the bacteria to bypass the critical steps in cell wall formation that are susceptible to antibiotics (Wilhelm, 1991).

1.1.4 *E. faecalis* Resistance

Many studies have been conducted over the past two to three decades to demonstrate how these pathogens adapt to various stress situations, which essentially involves the production of various genes that change the metabolism of microorganisms to create energy (Jett et al., 1994). When it comes to metabolism, enterococci are extremely adaptable bacteria that are resistant to extremes in temperature, pH, ionising radiation, high metal concentrations, and even certain medications (Paulsen et al., 2003). *E. faecalis* can withstand conditions with a pH range of 3.5-11.1 and high salt concentrations at temperatures as high as 60 degrees. Due to the fact that this tolerance affects many diseases and is taken into account specifically when testing for foodborne pathogens, *E. faecalis* pathogenicity and resistance have drawn widespread attention (Demple, 1991; Olson, 1993).

Bacteria that cause common or serious illnesses have gradually, and to varied degrees, become resistant to every new antibiotic that enters the market. One of the major public health issues of the twenty-first century is antimicrobial resistance (AMR), which poses a threat to the effective prevention and treatment of an expanding number of infections caused by bacteria, parasites, viruses, and fungi that are no longer susceptible to the conventional medications used to treat them (Prestinaci et al., 2015). A wide variety of resistance genes are present in the enterococcus species, and they can be exchanged. According to estimates, enterococci are the third most frequent cause of NIs and health care-associated BSIs and account for 25–50% of hospitalised patients' death rates. Additionally, concurrent multiple resistance mechanisms cause the creation of multi- or pan-resistant Enterococci, which do not react to the usual first-line antibiotics, raising the rates of morbidity and death and ultimately placing a greater cost burden on hospitals and patients (Jabbari Shiadeh et al., 2019).

Enterococci are resistant to cell-wall active substances like vancomycin and β-lactam antibiotics, which are typically bactericidal in nature. According to resistance, the antibiotic can inhibit bacteria at clinically viable quantities, but killing them requires concentrations that are far higher than the inhibitory concentration (Kristich et al., 2014).

1.1.5 *E. faecalis* Metabolism

As they can metabolise more than 30 different sugars, these bacteria are particularly effective at breaking down sugars (Sokatch & Gunsalus, 1957). This disease-causing bacteria, *E. faecalis*, can also metabolise glycerol, which is involved in a crucial process for lipid production and may be used as an energy source (Bizzini et al., 2009). During its proliferation, *E. faecalis* promotes the homo lactic acid fermentation of various carbohydrates and glycerol, which produces lactate as a byproduct (Feldman-Salit et al., 2013). NAD+ is created from NADH in this reaction, which takes place during glycolysis, by reducing pyruvate in the presence of the enzyme lactate dehydrogenase (LDH). LDH1 and LDH2 are two distinct LDH isoforms produced by the *E. faecalis* genome, and they resemble LDH-A and LDH-B of *Lactococcus lactis* (Bongers et al., 2003). However, LDH1 is more effectively transcribed in these two in relation to culture proliferation and is in charge of the majority of lactate synthesis (Mehmeti et al., 2012). Several studies have demonstrated that when the LDH gene is mutated in *E. faecalis*, lactate production decreases by about 25%, with the majority of the decrease being caused by the LDH1 mutations. By switching homolactic fermentation to heterolactic fermentation by overexpressing some genes and supressing other genes (M. Jönsson et al., 2009), leading to the formation of different acids, these additional pathways for producing energy in *E. faecalis* were also demonstrated. However, they

weakened the bacteria and had an impact on their pathogenicity and virulence (Richardson et al., 2008).

1.2 Lactate Dehydrogenase

LDH is an essential enzyme in anaerobic metabolism and falls under the category of oxidoreductases, with EC 1.1.1.27 as its enzyme commission number. Its main role is to facilitate the reversible conversion of lactate to pyruvate while simultaneously reducing NAD+ to NADH and vice versa. (Farhana & Lappin, 2022).

It speeds up the reaction by 14 times by synchronizing the inter-conversion of pyruvate to lactate and NADH to NAD+. The chemical reaction involves transferring a hydride ion from NADH to pyruvate at its C2 carbon. The molecular mechanism starts with NADH binding to the enzyme, where several active site residues are involved. Subsequently, lactate binds through interaction with NADH ring and LDH residues, and the hydride transfer occurs in both directions, forming two tertiary complexes, LDH-NAD+-lactate and LDH-NADH-pyruvate. (Shi & Pinto, 2014). In the LDH catalyzed reaction, pyruvate is dissociated from the enzyme before NAD+. The rate of dissociation of NADH and NAD+ is the rate-limiting step in this reaction. The final conversion of pyruvate to lactate, leading to the regeneration of NAD+, is thermodynamically favored (Spriet et al., 2000).

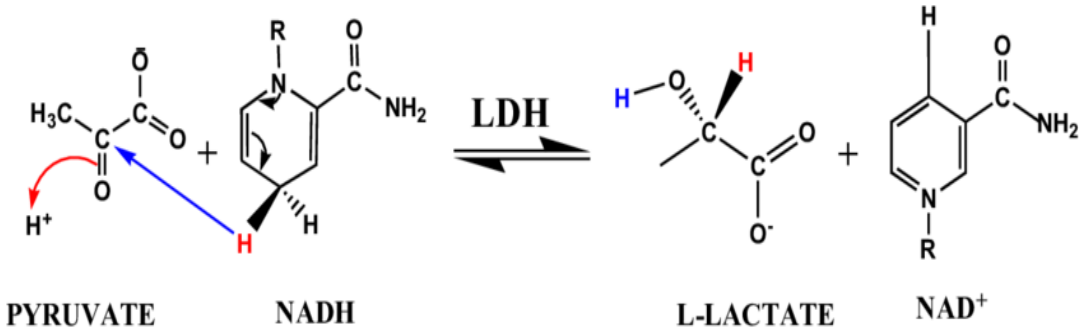


Figure 3 Lactate dehydrogenase metabolic pathway (Khan et al., 2019)

1.2.1 Lactate dehydrogenase and *E. faecalis*

Enterococcus faecalis is a bacterium that primarily utilizes the Embden-Meyerhoff-Parnas pathway (glycolysis) to break down glucose or similar carbohydrates. It is a lactic acid bacterium that undergoes homo fermentation. (Brown & Wittenberger, 1971). Under these conditions, lactate is the primary or main end product of fermentation. (M. Jönsson et al., 2009; Rana, 2012). *E. faecalis* has two cytosolic lactate dehydrogenase enzymes encoded by the ldh-1 and ldh-2 genes, which facilitate the reduction of pyruvate to lactate. This process helps in the regeneration of NADH to NAD+ and allows glycolysis to continue (Leboeuf et al., 2000). LDH-1 is the main contributor to the activity, while LDH-2 has a negligible effect (Maria Jönsson et al., 2009; Rana, 2012).

1.3 Oxamate

Oxamate, the salt of oxamic acid, is a compound with a similar structure to pyruvate that can competitively bind to LDH and inhibit its activity. This binding mechanism effectively stops the conversion of pyruvate to lactate (Zhai et al., 2013). This simple organic compound is water-soluble and has a molecular formula of $C_2H_2NO_3^-$

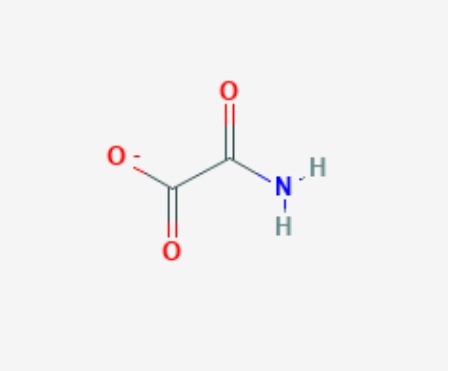


Figure 4 Chemical structure of oxamate

LDH is inhibited by oxamate through competitive binding with pyruvate, resulting in the formation of an inactive complex with the enzyme. Oxamic acid binds to the

NADH-LDH complex, leading to closure of the active site of LDH and its inhibition. LDH is overexpressed in malignant cells and tissues, which is associated with a poor prognosis in various cancers. The conversion of pyruvate to lactate requires NAD+ regeneration from NADH, which is crucial for glyceraldehyde 3-phosphate dehydrogenase to sustain glycolytic ATP synthesis. This reaction can also occur in certain benign cells during periods of rapid proliferation and hypoxia. However, glycolysis is the preferred mode of ATP synthesis in malignant cells, making it a viable target for cancer therapy. Oxamate inhibits LDHA, reducing ATP production in cancer cells and increasing the production of reactive oxygen species (ROS). While low levels of ROS may promote cancer cell proliferation, high levels can induce mitochondrial damage and kill cancer cells. (Altinoz & Ozpinar, 2022).

CHAPTER#2 LITERATURE REVIEW

2.1 Infections caused by *E. faecalis*

2.1.1 Endocardidtis

Endocarditis is a severe infection that arises when harmful microorganisms like bacteria infiltrate the bloodstream and attach to the inner lining of the heart chambers or heart valves. This can result in inflammation, scarring, and damage to the heart valves. If left untreated, this can lead to heart failure, which is a potentially life-threatening condition.

Endocarditis caused by *E. faecalis* may exhibit a range of symptoms, including fever, fatigue, joint and muscle pain, and a heart murmur. Other indications of the condition include shortness of breath, chest pain, and a persistent cough. However, these symptoms may not be specific to endocarditis, and the condition may be difficult to diagnose.

Therefore, it is crucial to seek medical attention if any of these symptoms are present, particularly if you have a history of heart disease, intravenous drug use, or recent surgical procedures. Early detection and appropriate treatment are essential in preventing severe complications and improving the prognosis of endocarditis.

The treatment approach for endocarditis induced by *E. faecalis* primarily involves antibiotics. However, the selection of antibiotics to be used is dependent on the susceptibility of the particular strain of *E. faecalis* that caused the infection. Commonly, Vancomycin is utilized to manage enterococcal infections.

In some instances, surgery may be deemed necessary to repair or replace the heart valves that may have been damaged by the infection. It is important to understand that endocarditis is a severe condition, and prompt diagnosis and treatment are essential. If

left untreated, the condition can lead to life-threatening complications such as heart failure, stroke, and emboli.

It is, therefore, vital to seek medical attention as soon as possible if you experience symptoms of endocarditis, particularly if you have a history of heart disease, intravenous drug use, or recent surgical procedures. Early diagnosis and treatment are crucial in preventing severe complications and improving the prognosis of the condition (Seby et al., 2022).

2.1.2 Urinary tract infections

A urinary tract infection (UTI) is a type of infection that can affect any part of the urinary system, which includes the ureters, bladder, urethra, and kidneys. Enterococcus faecalis is a specific type of bacteria that can cause UTIs, particularly infections of the bladder and urethra.

UTIs caused by *E. faecalis* can manifest in various ways, such as a frequent urge to urinate, a burning sensation while urinating, and cloudy or pungent urine. Additionally, lower abdominal pain is another symptom that might be present. However, it's important to note that in some cases, there may be no symptoms at all.

It's important to seek medical attention if you experience any of the symptoms mentioned above. This is especially crucial if you have a history of UTIs, are pregnant, have diabetes, or have any condition that weakens your immune system. Early diagnosis and treatment can prevent complications that may arise from untreated UTIs caused by *E. faecalis* (Flores-Mireles et al., 2015).

When it comes to treating UTIs caused by *E. faecalis*, antibiotics such as ampicillin, amoxicillin, or vancomycin are commonly prescribed. However, the selection of

antibiotics used may vary depending on the susceptibility of the particular strain of *E. faecalis* causing the infection.

The course of treatment typically lasts between 7 to 14 days, and it is crucial to complete the entire course of antibiotics even if the symptoms disappear before the course ends. This is to prevent the development of antibiotic resistance, which can make future infections more difficult to treat.

It's also essential to drink plenty of water and urinate frequently to flush bacteria out of the urinary tract. Additionally, avoiding the use of bubble baths, spermicides, and diaphragms can help reduce the risk of developing a UTI (Foxman, 2010).

If you experience symptoms of a UTI, it's essential to seek medical attention promptly. This is particularly important if you have a history of UTIs, are pregnant, have diabetes, or have any condition that weakens your immune system. Early diagnosis and treatment can prevent complications that may arise from untreated UTIs caused by *E. faecalis*.

If a UTI caused by *E. faecalis* recurs or persists, alternative treatments may be necessary. These can include extending the course of antibiotics, increasing fluid intake, or administering a low-dose antibiotic regimen (Gupta & Bhadelia, 2014).

2.1.3 Prostatitis

Prostatitis refers to an inflammation of the prostate gland, a small gland situated near the bladder in men that produces fluid responsible for transporting and nourishing sperm. Although relatively uncommon, Enterococcus faecalis is a type of bacteria that can cause prostatitis.

When caused by *E. faecalis*, prostatitis may present with symptoms such as pelvic pain, pain or discomfort during urination or ejaculation, difficulty urinating, and flu-like symptoms including fever and chills. Some individuals with prostatitis may also

experience lower back pain or a sense of fullness in the rectum (Coker & Dierfeldt, 2016).

When prostatitis is caused by *E. faecalis*, it is typically treated with antibiotics, including ampicillin, amoxicillin, or vancomycin. The specific antibiotic used will depend on the susceptibility of the *E. faecalis* strain causing the infection. Treatment may last from 4 to 6 weeks.

To ensure effective treatment, it is crucial to adhere to the physician's instructions regarding antibiotic use, including duration and frequency of administration. Additionally, dietary and lifestyle changes may be recommended to help prevent a recurrence of the infection.

To address recurrent or persistent cases of prostatitis caused by *E. faecalis*, doctors may consider other measures including a longer course of antibiotics, increasing fluid intake or using a low-dose antibiotic regimen. Furthermore, people with underlying medical conditions such as diabetes may be more susceptible to developing prostatitis caused by *E. faecalis* and may need a more aggressive treatment approach.

It's worth noting that in some cases, prostatitis caused by *E. faecalis* may not cause any symptoms, which highlights the importance of getting tested if you are at risk of developing prostatitis or have a history of recurrent urinary tract infections. Following the doctor's advice regarding the use of antibiotics, lifestyle changes, and monitoring for potential recurrence is also crucial for effective management of prostatitis caused by *E. faecalis* (Lipsky et al., 2010).

2.1.4 Intra-abdominal infections

Intra-abdominal infections refer to infections that occur within the abdominal cavity, which houses several vital organs including the stomach, intestines, liver, and pancreas.

Although less common than other bacteria, Enterococcus faecalis is still known to cause intra-abdominal infections.

Infections within the abdominal cavity can be caused by several factors, including the perforation of the gastrointestinal tract, surgical procedures, and the spread of infection from other organs. Intra-abdominal infections can be classified into two categories: primary peritonitis and secondary peritonitis. Primary peritonitis occurs when an infection develops within the peritoneal cavity without an obvious source, whereas secondary peritonitis occurs as a result of a perforated viscus or traumatic injury to the peritoneum (Nicoletti et al., 2009).

Intra-abdominal infections caused by *E. faecalis* can present with various symptoms such as abdominal pain, fever, nausea, vomiting, and diarrhea. In severe cases, sepsis may occur, which is a serious condition characterized by a systemic inflammatory response to an infection. It is important to seek medical attention promptly if you experience any of these symptoms.

Typically, antibiotics such as ampicillin, amoxicillin, or vancomycin are used to treat intra-abdominal infections caused by *E. faecalis*. The specific antibiotic prescribed will depend on the susceptibility of the specific strain of *E. faecalis* causing the infection. It is important to follow the prescribed treatment regimen and complete the full course of antibiotics, even if the symptoms disappear.

In some cases, surgical intervention may be necessary to remove the source of the infection. This may involve draining abscesses or removing infected tissue. Your healthcare provider will determine the appropriate treatment plan based on the severity of the infection and other individual factors.

Prompt and effective treatment is crucial for intra-abdominal infections caused by *E. faecalis* since it can be life-threatening if left untreated. Early diagnosis and treatment can help prevent complications such as peritonitis, sepsis, and multiple organ failure.

Close monitoring and intensive care may be necessary for patients with intra-abdominal infections caused by *E. faecalis* during treatment. Individuals with underlying medical conditions such as diabetes may be at a higher risk of developing these infections and may require more aggressive treatment.

It is important to seek medical attention immediately if any symptoms of intra-abdominal infections are experienced. In addition, preventive measures such as proper hygiene, avoiding high-risk activities, and maintaining a healthy lifestyle may help reduce the risk of developing intra-abdominal infections caused by *E. faecalis* (Swenson et al., 2009).

2.1.5 Cellulitis

Cellulitis is a skin infection that can occur when bacteria, including Enterococcus faecalis, enters the skin through a break or wound. This type of infection typically causes a painful, swollen, and red area of skin that feels warm to the touch. The infected area may also appear firm and hard, and red streaks may be visible spreading from the site of the infection.

Symptoms of cellulitis caused by *E. faecalis* may include flu-like symptoms such as fever, chills, headaches, and muscle aches, as well as fatigue. The affected area of the skin may also be tender and swollen, and it may feel warm to the touch. In some cases, redness, warmth, and tenderness may also be present.

To treat cellulitis caused by *E. faecalis*, antibiotics like penicillin or ampicillin are typically used, depending on the strain's susceptibility. The treatment usually lasts for

7 to 10 days. It is important to follow the doctor's instructions carefully regarding the dosage and duration of antibiotics, as well as any lifestyle changes recommended to prevent the recurrence of the infection.

If the infection is persistent or recurrent, other measures such as a longer antibiotic course, increasing fluid intake, or a low-dose antibiotic regimen may be used. Furthermore, individuals with certain medical conditions such as diabetes are at higher risk of developing cellulitis caused by *E. faecalis* and may require more aggressive treatment.

Therefore, it is important to seek medical attention promptly if you suspect that you have cellulitis, and to follow the prescribed treatment plan to ensure a full recovery.

2.1.6 Wound infections

An infection of a wound caused by Enterococcus faecalis happens when the bacteria enter a break or cut in the skin. This type of bacteria is commonly found in the gastrointestinal tract and is known to cause nosocomial infections, and they are also known to resist several antibiotics.

Symptoms of a wound infection caused by *E. faecalis* may include warmth, pain, redness, and tenderness around the wound, as well as fever, chills, and flu-like symptoms. In some cases, the wound may also be swollen, and there may be drainage of pus or other fluids from the wound (Rajkumari et al., 2014).

Treatment for a wound infection caused by *E. faecalis* typically involves antibiotics, such as ampicillin, amoxicillin, or vancomycin, depending on the susceptibility of the specific strain of *E. faecalis* causing the infection. It is important to follow the doctor's instructions regarding the use of antibiotics, how long to take them, and any dietary and lifestyle changes that may be recommended to help prevent recurrence of the infection.

Wound infections caused by Enterococcus faecalis can be life-threatening if not promptly and effectively treated, therefore early diagnosis and treatment are crucial in preventing complications such as sepsis, cellulitis, and multiple organ failure. To avoid contamination, it's important to keep the wound clean and dry, and to change dressings as directed by a healthcare provider.

Additionally, individuals with certain medical conditions, such as diabetes, may be at higher risk for wound infections caused by *E. faecalis* and may require more aggressive treatment. It's important to properly clean and disinfect the wound before applying dressings and avoid exposing the wound to dirty or contaminated environments.

Finally, the wound should not be closed until it has completely healed to prevent trapping bacteria and reducing the risk of infection (Rajkumari et al., 2014)

2.1.7 Bacteraemia

Concurrent bacteraemia caused by Enterococcus faecalis is a condition where *E. faecalis* bacteria is found in the bloodstream alongside an existing infection or condition. *E. faecalis* is a type of bacteria that is typically found in the gastrointestinal tract and is known to cause nosocomial infections. Moreover, this bacteria has the ability to resist several antibiotics, which can make treatment more challenging. Symptoms of concurrent bacteraemia caused by *E. faecalis* can include fever, chills, and flu-like symptoms, as well as fatigue, weakness, and muscle aches. Additionally, people with this condition may also experience symptoms related to the underlying infection or condition, such as skin infections, urinary tract infections, endocarditis, or intra-abdominal infections. Early diagnosis and treatment are crucial to preventing complications associated with concurrent bacteraemia, such as sepsis or multiple organ failure.

To diagnose concurrent bacteraemia caused by *E. faecalis*, the usual method involves performing a blood culture test to detect the presence of bacteria in the bloodstream. Besides blood culture, doctors may also use additional diagnostic tools like blood cell count, imaging tests, and other laboratory tests to identify the underlying infection or medical condition.

To treat concurrent bacteraemia caused by *E. faecalis*, antibiotics like ampicillin or vancomycin are typically prescribed, based on the susceptibility of the specific strain of *E. faecalis* causing the infection. Additionally, treating the underlying infection or condition may also be necessary. It's important to note that if left untreated, concurrent bacteraemia caused by *E. faecalis* can be life-threatening, particularly in people with underlying health conditions. Early diagnosis and treatment are therefore crucial to avoid complications such as sepsis, cellulitis, and multiple organ failure.

It's important to monitor the patient for any signs of deterioration and ensure that the underlying condition is being treated effectively. Certain medical conditions such as diabetes may increase the risk of developing concurrent bacteraemia caused by *E. faecalis* and may require more aggressive treatment (Dahl et al., 2019).

2.1.8 Meningitis

Enterococcus faecalis meningitis is a severe and infrequent infection that occurs when the bacteria invade the meninges, the protective layers that surround the brain and spinal cord. Enterococcus faecalis is a type of bacteria that is commonly present in the gastrointestinal tract and is known to cause hospital-acquired infections. Additionally, it has the ability to resist multiple antibiotics.

Symptoms of meningitis caused by *E. faecalis* may manifest as fever, headache, stiff neck, sensitivity to light, confusion, and changes in mental status, as well as nausea,

vomiting, drowsiness, and seizures. In severe cases, this infection can cause permanent neurological damage, hearing loss, and death. Patients with a weakened immune system, such as those with HIV, cancer, chronic disease, or who have undergone neurosurgery, are at an increased risk of developing meningitis caused by *E. faecalis*.

To diagnose meningitis caused by *E. faecalis*, doctors typically perform a lumbar puncture to collect cerebrospinal fluid (CSF). This sample is then analyzed for the presence of bacteria and other abnormal findings.

Antibiotics such as ampicillin or vancomycin are commonly used to treat meningitis caused by Enterococcus faecalis. The specific type of antibiotic prescribed depends on the susceptibility of the particular strain causing the infection. Supportive care, including fluid replacement, pain management, and monitoring for complications, may also be necessary.

It's important to note that meningitis that is caused by *E. faecalis* can be fatal, especially in individuals with underlying medical conditions. Early diagnosis and treatment are essential in preventing complications, such as brain damage, hearing loss, and death. Monitoring for signs of deterioration and treating any underlying conditions are also important. Those with certain medical conditions, such as diabetes, may require more aggressive treatment (Zhang et al., 2021).

2.2 Therapeutic Approach for *E. faecalis* resistance

2.2.1 LDH- An effective drug target

Various test were performed by creating mutant LDH-deficient strains to explain that the fitness and virulence of LDH-deficient strains is majorly diminished in stressing conditions (Rana et al., 2013). The ability of *E. faecalis* to persist in hospital environments and cause infections is attributed to its intrinsic ruggedness, which can

be reduced by inhibiting its ability to maintain redox balance via the LDH reaction. In addition, this inhibition also results in decreased fitness during infection, suggesting that LDH may be a promising drug target. A drug that targets LDH could have wider applications. For instance, glycopeptide antibiotics like vancomycin work by binding to pentapeptide precursors' C-terminal D-alanyl-D-alanine, preventing trans-glycosylation and trans-peptidation in cell wall assembly. Resistance to vancomycin results from the synthesis of modified peptidoglycan precursors ending in D-alanyl-D-lactate, to which glycopeptides exhibit low binding affinities. The synthesis of D-lactate is catalyzed by VanH dehydrogenase, which is a D-LDH that converts pyruvate into D-lactate. (Leclercq & Courvalin, 1997; Rana et al., 2013). Disabling this enzyme would work against glycopeptide resistance. (Hirschhaeuser et al., 2011; Sattler et al., 2010).

2.3 Oxamate as an anti-cancer agent

The metabolism of cancer cells is reprogrammed to support their growth, survival, proliferation, and maintenance. One of the main changes in metabolism is the uptake of glucose and its conversion to lactate through a process of fermentation (Warburg effect) (Liberti & Locasale, 2016).

Oxamate is a widely-used drug in the fight against cancer. It has been shown to effectively block the glycolytic pathway by inhibiting LDH, which in turn prevents the production of lactate. Cancer cells have a unique ability to generate energy through aerobic glycolysis, even in the presence of oxygen. LDH plays a crucial role in this process by catalyzing the conversion of pyruvate to lactate. Although oxamate has shown promise as an anticancer agent, the precise mechanism by which it operates is not yet well understood (Zhao et al., 2015).

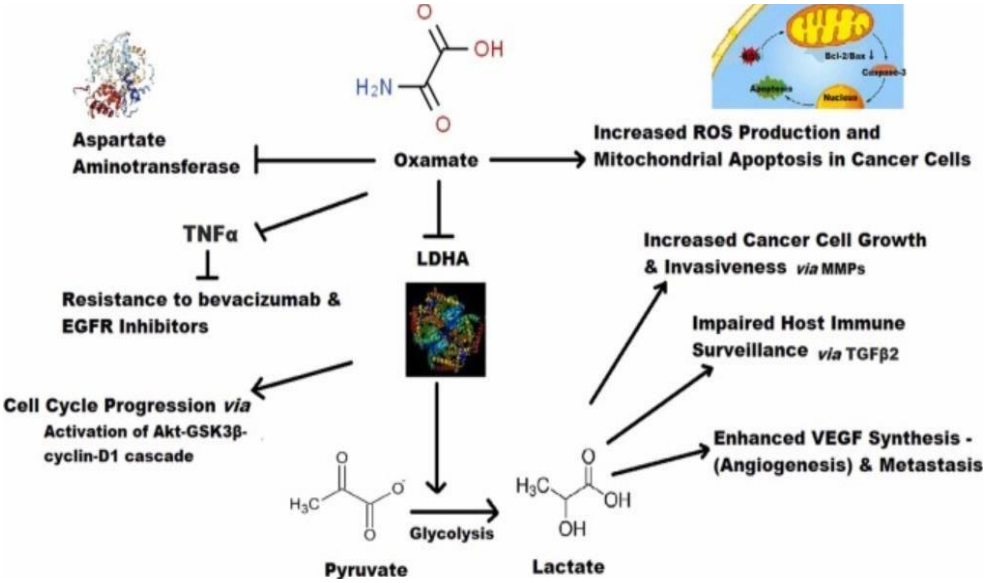


Figure 5 Mechanism of action of oxamate as an anti-cancer agent (Altinoz & Ozpinar, 2022)

2.3.1 Glycolytic metabolism in tumor cells

In tumors of all types, the Warburg effect is present, which refers to the dominance of glycolysis as the primary energy metabolism of cancer cells even in the presence of sufficient oxygen (Burgess, 2012; Sanderson & Locasale, 2018). Tumor cells take up significant amounts of glucose, and convert pyruvate to lactic acid using lactate dehydrogenase A (LDH-A), which is a crucial enzyme in the glycolysis process. Simultaneously, they also oxidize nicotinamide adenine dinucleotide hydride (NADH) to nicotinamide adenine dinucleotide (NAD+) (Flores et al., 2019). The glycolytic metabolism of tumor cells results in competition for scarce nutrients within the tumor

microenvironment and causes a shortage of nutrients for stromal and immune cells (Lundø et al., 2020; Polyzos et al., 2019). The tumor microenvironment is affected by the main metabolites produced by tumor cells. The transportation of a significant amount of lactic acid, generated by glycolysis, out of the tumor cells through the monocarboxylate transporter (MCT) results in acidification of the tumor microenvironment, which further stimulates the secretion of angiogenesis and vascular endothelial growth factor (VEGF) (Lee et al., 2015; Zhang & Wang, 2020). The excessive lactic acid produced by tumor cells can elevate the concentration of lactic acid in the tumor microenvironment and hinder the efflux of lactic acid generated by the glycolysis of T lymphocytes. This leads to the acidification of the intracellular environment of T lymphocytes, hindering their activation and promoting the evasion of the tumor from the immune system (Ganapathy-Kanniappan, 2017; Vinasco et al., 2019). Thus, lactic acid, produced as a byproduct of glycolysis, not only aids in tumor cell invasion, metastasis, and angiogenesis, but also facilitates immune evasion by acidifying the tumor microenvironment. Given its crucial role in cancer metabolism, lactic acid metabolism and its key enzymes are viewed as potential targets for cancer treatment (Martinez-Outschoorn et al., 2017). Targeting LDH-A as an anticancer strategy is attractive because it is not a critical enzyme in normal cell metabolism, which

suggests that selective inhibitors of LDH-A may have minimal side effects.

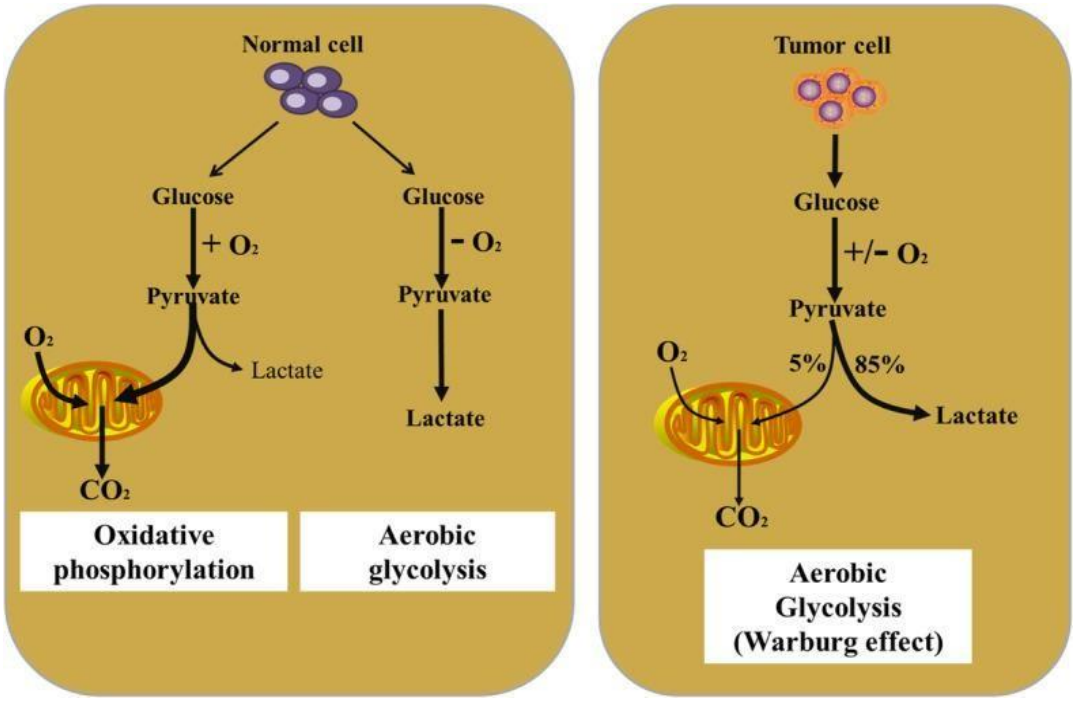


Figure 6 Regulation of glucose metabolism in cancer cells (Xia et al., 2021)

2.4 Oxamate inhibition of LDH in various types of cancers

Researches done on different types of cancers showing oxamate inhibition of LDH are as following

2.4.1 Attenuation of Medulloblastoma

Oxamate is an effective inhibitor of LDHA and aerobic glycolysis, which slows down ATP generation and limits medulloblastoma cell proliferation. About 30% of medulloblastomas are metastatic and have a poorer prognosis, and previous research in other cancers suggests that lactate is a predictor of metastasis. Live cell imaging experiments demonstrate that oxamate substantially suppresses medulloblastoma cell migration. Therefore, inhibiting LDHA and lactate production using oxamate shows

promise in cancer treatment. However, the concentration of oxamate required to produce significant effects is too high for clinical use (Valvona & Fillmore, 2018).

2.4.2 Action on Glial Brain Tumors

Glial tumor cells have increased expression of LDHA, resulting in the synthesis of LDH IV and V enzymes, which convert pyruvate to lactate. Additionally, the increased production of LDHB leads to the synthesis of LDH I, II, and III, catalyzing the oxidative conversion of lactate to pyruvate. In vivo experiments with C6 glioblastoma implanted into rat brains showed that oxamate can significantly reduce lactate levels as determined by MRS studies (Altinoz & Ozpinar, 2022).

2.4.3 Suppression of Nasopharyngeal carcinoma

Oxamate, which inhibits LDH, disrupted energy metabolism and reduced ATP production in NPC cells. To investigate alterations in cell cycle distribution after oxamate treatment, the proportions of cells in the G0/G1 phase were analyzed, and they were found to decrease. However, the S phase fractions remained unchanged in both cell lines. Notably, the percentage of cells in the sub-G1 phase significantly increased after treatment with oxamate. Moreover, oxamate induced apoptosis in cancer cells via the mitochondrial pathway and increased sensitivity to ionizing radiation (Zhai et al., 2013).

2.4.4 Action in thoracic cancers

Oxamic acid, another LDH-A inhibitor, has been shown to increase the in vitro anti-proliferative activity of certain tyrosine kinase inhibitors. However, the activity of tyrosine kinase inhibitors can be reduced by high levels of LDH. These inhibitors work by competing with ATP for binding to the ATP binding site. Under hypoxic conditions, LDH catalyzes the final step of anaerobic glycolysis, converting NADH and pyruvate

into NAD+ and lactate. Inhibiting LDH decreases anaerobic glycolysis in cancer cells, leading to a reduction in ATP production. This decrease in ATP reduces the competition of ATP with tyrosine kinase inhibitors and enhances their efficacy. This could be the reason why high serum LDH levels are associated with increased effectiveness of tyrosine kinase inhibitors (Comandatore et al., 2022).

2.4.5 Inhibition of proliferation of gastric cancer

The dose-dependent effect of oxamate on cell proliferation was observed in the presence of glucose. No morphological changes were observed, but the number of cells decreased with oxamate treatment. A 50% decrease in lactic acid production was observed after 4 hours of oxamate exposure, and the effective concentration for suppression was similar to previous reports. These results indicate that oxamate can inhibit glycolysis in gastric cancer cells, resulting in reduced cell invasion and apoptosis (Liu et al., 2015).

Furthermore, studies have shown that oxamate is effective in reducing the proliferation of breast cancer, cervical cancer, and liver cancer cells. (Stone et al., 2019 {Cassim, 2018 #54)}.

2.4.6 Action in cervical cancer

Oxamate has been demonstrated to exert its antitumor effects in cervical cancer by inhibiting lactate dehydrogenase (LDH), a key enzyme involved in the metabolic reprogramming of cancer cells. This inhibition disrupts the cancer cells' energy production and survival.

Moreover, Oxamate can also inhibit the Warburg effect, which is associated with the development and progression of cervical cancer, by reducing glucose uptake and lactate production in cancer cells.

In vitro studies have confirmed that oxamate can effectively inhibit the growth of cervical cancer cells and promote apoptosis, resulting in a reduction in the rate of cell proliferation (Stone et al., 2019).

2.4.7 Inhibition in NSCL cancer

The overexpression of LDH-A in NSCLC is linked to poor prognosis and greater resistance to chemotherapy. In vitro studies have demonstrated that oxamate can efficiently hinder LDH-A activity in NSCLC cells. Oxamate has been shown to impede the proliferation of NSCLC cells by decreasing their rate of cell growth and increasing the rate of apoptosis (Li et al., 2013).

2.4 Toxicity of Oxamate

Oxamate is generally regarded as safe when used at the recommended doses. The compound inhibits AAT, which is a widely distributed enzyme that relies on pyridoxal phosphate, and works in conjunction with malate dehydrogenase to facilitate the NADH shuttle, which links glycolysis and mitochondrial energy production. Therefore, it has been suggested that inhibiting AAT with oxamate may have detrimental effects on glucose metabolism in muscle, adipose, and hepatic tissues. (Altinoz & Ozpinar, 2022). Although oxamate is generally considered safe when used at recommended concentrations, prolonged exposure to high doses of the drug may result in adverse effects. Reported side effects include:

- **Gastrointestinal discomfort:** Oral consumption of oxamate can lead to symptoms such as nausea, vomiting, and diarrhea.
- **Nephrotoxicity:** Excessive amounts of oxamate can result in renal impairment, potentially leading to kidney failure.

- **Cardiotoxicity:** Heart failure can be a potential consequence of administering high doses of oxamate, as it may cause damage to the heart.
- **Hematotoxicity:** Anemia or other blood disorders may occur as a result of high doses of oxamate.

It should be emphasized that the toxicity of oxamate can vary depending on the person and the intended use. As a result, it is essential to adhere to the recommended usage guidelines and to keep an eye out for any negative consequences.

2.5 Effect of different physiological stresses on bacterial growth

2.5.1 Sodium dodecyl sulphate (SDS)

The cell membrane and cell wall, which serve as the first line of defense for each cell, are impacted by Sodium Dodecyl Sulphate (SDS), a common household detergent. It disrupts cell membranes, sets off stress responses such Cell Wall Integrity (CWI) signaling, and prevents the growth of new cells. (Schroeder & Ikui, 2019).

2.5.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide, a topical antibiotic intended to treat wounds, eliminates bacteria by local oxygen production and oxidative bursts. According to reports, H2O2 is a biochemically active molecule produced by a variety of cells that alters intracellular redox balance, which regulates the activity of various signaling transduction pathways, changes in membrane potential, and the production of new molecules. (Zhu et al., 2017).

2.5.3 Ethanol

Alcohol has been found to have many inhibitory effects on microbes. Growing research suggests that its toxicity affects the cell membrane. Numerous studies demonstrate that even at modest concentrations, ethanol exhibits a fluidizing effect on cellular

membranes. When ethanol arrives, growth happens, and this adaptation leads to an increase in ethanol tolerance (Alexandre et al., 1994).

2.5.4 Dimethyl sulfoxide (DMSO)

Although the exact mechanism of DMSO's bacteriostatic activity is unknown, it has been proposed that bacterial membrane infiltration and disruption are the main causes of DMSO's antimicrobial activities (Ansel et al., 1969). Bacteria are poisoned by the sulphoxide component of DMSO, which has a sulphur S=O bond. (Awan et al., 2020).

2.5.5 Glucose

An osmotic imbalance is produced in bacteria when 10% glucose is added. An initial glucose level of 5% is already very close to the upper limit of osmotic tolerance because the ideal osmotic pressure for E. coli for sustaining its physiologic activity is around 300 mosmol (Xiao et al., 2017). Osmotic stress can lower cellular production and even result in cell inactivation. (Varela et al., 2004)

2.5.6 Hypochlorous acid (HOCl)

HOCl is thought to be essential for the eradication of germs despite the fact that its mode of activity has yet to be well investigated. Furthermore, despite the fact that numerous in vitro studies have shown that HOCl is a general or detrimental oxidant (particularly at high doses) (McKenna & Davies, 1988)

2.6 Effect of stressors on *E. faecalis* and *E. faecalis* resistance

E. faecalis is regarded as a particularly "tough" bacterium since it can endure stressful conditions that would kill other bacteria (Sherman, 1938). It can also endure being dried out on surfaces for months (Kramer et al., 2006). The capacity of *E. faecalis* non-growing cells to survive under fatal conditions is highly greater than that of growing cultures as a result of the development of various, nonspecific stress resistances.

Together, these bacteria's exceptional intrinsic toughness as well as resistance to drugs are thought to be the cause of their effectiveness in persisting and spreading throughout healthcare environments (Rana et al., 2013).

2.7 Stress sensitivity in bacteria with diminished LDH

It is observed experimentally that bacterial strains with reduced or deficient LDH production are generally more stress sensitive (Rana et al., 2013).

2.8 Computational Analysis for the Prediction of oxamate as a Potential Inhibitor of LDH Enzyme of *E. faecalis*

2.8.1 Bioinformatics resources

Bioinformatics is an interdisciplinary field that utilizes computer science tools such as chimera, ProSA web, Verify3D, and PyMOL to store, retrieve, organize, and analyze biological data. It plays a crucial role in analyzing genes, their expressions, interactions, and comparing them with other genes and molecules through simulations and modeling. Bioinformatics also provides evolutionary aspects (Dill et al., 1995; Joyce et al., 2015). Online databases such as PDB, NCBI, EMBL, Genbank, and KEGC are used for storing and maintaining vast biological data. The use of bioinformatics tools is necessary to conduct scientific research within a minimal time frame, solve and analyze complex problems with a low error rate. Human error chances are significantly higher than computational analysis, making bioinformatics tools indispensable (Ouzounis & Valencia, 2003).

2.8.2 Demand for the use of Bioinformatics resources

Bioinformatics has emerged as a vital interdisciplinary field of science that plays a significant role in solving complex biological problems, particularly in molecular biology and genomics. By using computer programming, bioinformatics allows for the

efficient study of genes, their regulation, and their interaction with other molecules during and after expression, making it an essential tool in scientific research. The vast amount of biological data, such as the human genome, protein sequence and structure, and metabolic pathways, cannot be handled by humans alone. Therefore, bioinformatics tools and software are used to control, organize, and manipulate large amounts of biological data. The idea of using computational techniques for biological applications was first suggested in the early 1970s when the first RNA sequence was published, followed by the Human Genome Project in the mid-1980s, which produced a massive amount of data that could not be easily analyzed and handled. To address this challenge, scientists proposed the use of bioinformatics resources, leading to the rapid emergence of this field. Today, complex software has been developed to solve many biological and other related problems (Zhang et al., 2006).

2.8.2.1 Docking

To simulate the interaction between molecules like protein-enzyme or protein-protein, computational docking is used. In the molecular biology field, understanding the association of proteins with small molecules is crucial for regulating protein function. By performing docking, software can predict the capability of proteins and ligands to bind together and form stable complexes. If two molecules have a higher binding affinity or a better ability to form a complex, this can help predict the strength and type of bond between them (Kitchen et al., 2004). Docking is useful in determining the role of various molecules in biochemical processes and in rational drug design (Langer & Hoffmann, 2001).

2.8.2.2 Binding Confirmations of Docking Structures

Molecular docking is a useful tool for determining binding affinities between ligands and proteins. The strength and type of interaction between the two molecules can be

predicted by the binding affinity, with higher binding energies indicating a stronger bond.

However, different visualization software may have varying cutoff values, which can result in differences in the number of hydrogen bonds observed in the same intermolecular complex.

Top Hits of Docking of Molecules from Literature search

Molecules	**S-Scores**
Gossylic nitrile	-8.9
Chimeric Type Inhibitor-1	-8.5
Isodolinyl	-8.6
Sulphonamide based quinolone derivative	-8.1
N-Hydroxy Indole derivative	-7.8
Gossylic lactone	-7.8
2-3-Hydroxynaphthoic acid	-7.7
Dideoxy Gossylic acid	-7.4
Glucose derivative	-7.4
Cyclohex-2-enone	-7.1

Diacid malonate scaffold-based inhibitor	-7.0
Gossypol	-6.8
Galloflavin	-6.7
Sodium Oxamate	**-4.5**

Table 2 : Highest hits of binding energies of some of the ligands that have been searched through literature and docked against modelled protein of LDH.

The above table confirms that oxamate showed the binding affinity with LDH protein so it can be further analysed for its potential in killing the bacteria by inhibiting the LDH enzyme.

Aims of research

E.faecalis is the second most dangerous bacterium that can cause a range of infections such as nosocomial infections, UTIs, soft tissue infections, bacteremia and endocarditis. It has shown resistance to several antibiotics, as well as high temperatures, pressure, radiation, and metal presence. One potential approach to combat this bacterium is to identify compounds that can inhibit its growth. In this study, oxamate will be examined using an in vitro method to observe its ability to inhibit the lactate dehydrogenase enzyme (LDH), which is the primary energy source for *E.faecalis*. This enzyme utilizes glucose and 20 other types of sugar to produce energy for the bacteria. By interacting with this molecule, oxamate could prevent the bacteria from producing energy, leading to its weakening and eventual death even with a small dose of antibiotic.

CHAPTER#3 MATERIALS AND METHODOLOGY

3.1 Materials

The *E. faecalis* Strain JH2-2 (derived from the parental strain JH2) was cultured in GM 17 media with MOPS (3-(N-morpholino) propane sulfonic acid) as the buffering agent. The M17 media was supplemented with 0.5% (wt./vol) of 30% glucose and the culture was maintained at 37 degrees Celsius. Oxamate 98+% (Alfa Aesar USA) and Lauria Bertani Broth (CM1018 OXOID) were also used.

The GM 17 media contained Universal Peptone 5g/L (HIMEDIA), Tryptic Soy Broth 5g/L (Merck Germany), Yeast Extract 2.5g/L (Sigma Aldrich), Beef Extract (Sigma Aldrich), Ascorbic Acid 0.5g/L (BDH AnalaR), Magnesium Sulphate 0.25g/L (GPR BDH), MOPS (3-(N-morpholino) propane sulfonic acid 42g/L (Sigma Aldrich), and Sodium Hydroxide Pellets (GPR BDH) to maintain pH between 7.1 to 7.3.

Glucose was added separately, sterilized by autoclave, and then added to the media by 0.5% w/v after cooling in a sterile environment.

Figure 7 GM17 Media in 10ml Test Tubes after ng autoclaving

3.2 Methodology

3.2.1 Bacterial isolation and culturing

Bacteria were isolated using the spreading and streaking method on Lauria Bertani Broth and LB Agar, as well as on GM17 Agar. Bacterial cultivation was carried out using GM17 Broth Media.

To prepare the glycerol tube for daily experiments, a fresh preculture of *E. faecalis* was obtained from a pure isolated colony on GM17 Agar, which was grown overnight in 10ml of GM17 Media in a 25ml test tube. Then, 1ml of the fresh bacterial preculture and 1ml of 0.5 percent glycerol solution were added to a 10ml GM17 Media in a 25ml test tube overnight. The glycerol tube was then sampled by drawing 50 microliters, which was inoculated into a 10 ml test tube containing GM17 medium. The mixture was incubated at 37 °C and 120 rpm overnight.

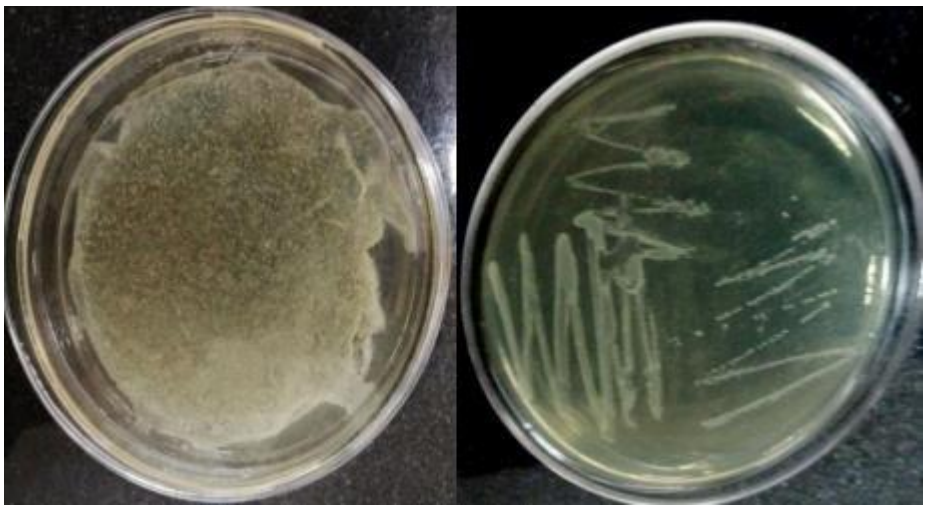


Figure 8 Spreading and streaking on LB agar

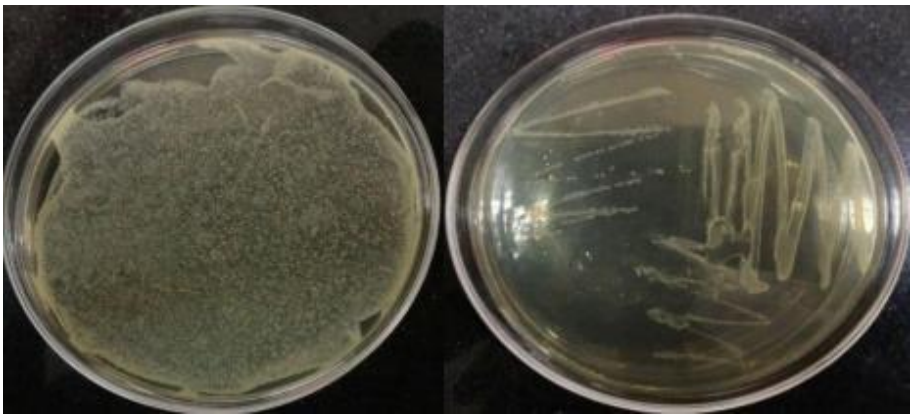


Figure 9 Spreading and streaking on GM17 Agar

3.2.2 Concentrations of Oxamate for Evaluation:

To evaluate the inhibitory effect on growth, eight different concentrations (5, 10, 25, 50, 75, 100, 150, and 200 micrograms per milliliter) of Oxamate were tested. The first tube containing the bacterial preculture with media served as a control, while the second tube contained 100 microliters of water, which was used as the solvent for the control. The third to tenth tubes were filled with different concentrations of the drug. The OD at 600nm was measured and recorded after one hour of incubation at 37 degrees Celsius and 120 revolutions per minute. The tubes were then incubated in a shaking water bath under the same conditions for seven hours, and the absorbance at 600 nm was measured every hour.

Figure 10 Stock solution of oxamate

3.2.3 Serial Dilution:

After reaching a concentration of 0.8 at 3 hours and 4 hours after drug for controls with media and preculture only, as well as for two different drug concentrations of 75 and 100 micrograms per milliliter, serial dilution was carried out to determine the minimum inhibitory concentration. The inhibition at 100 micrograms per milliliter was clearly

Figure 11 Serial dilution

3.2.4 Bacterial Cell Lysis and Lactate Dehydrogenase Activity Determination:

After an 8-hour incubation, 1ml was taken from each test tube and centrifuged at 4500 rpm for 5-10 minutes at 4 degrees Celsius. The pellets remained in their original Eppendorf tubes, while the supernatant was discarded into ten different Eppendorf tubes. The bacterial cells in the pellets were vortex-lysed with 100 microliters of Cell lysis buffer and 200 microliters of autoclaved distilled water. After that, the supernatant was centrifuged at 1500 rpm for ten minutes, and the resulting supernatant was used to conduct the LDH Assay. The LDH Assay kit protocol for the determination of Lactate

Dehydrogenase was followed using the (LDH-P FLUID 4+1) Kinetic Assay, in accordance with the manufacturer's instructions.

Figure 12 Supernatant collection

3.2.5 Absorbance on Microscale (Broth Dilution Method)

Fresh bacterial culture was prepared from an overnight preculture and incubated in a shaking water bath at 37°C. Eleven wells of a 96-well microplate were used for culturing, where well 1 served as a blank and well 2 as a control using GM17 medium and preculture. Culture media was added to wells 3 to 10.

The microplate was then placed in a microplate reader with an incubation temperature of 37°C and a kinetic loop of 3 hours with an interval of 1 hour. The absorbance was set at a wavelength of 600nm with continuous shaking. The initial absorbance result was calculated in the first minute, and three further absorbance values were calculated after a one-hour interval.

After three hours of incubation, the experiment was stopped when the OD reached 0.8. The plate was then removed, and drug solutions were added to each well except for wells 1 and 2, which served as blank and control, respectively. Wells 3 to 11 received different concentrations of Oxamate ranging from 5 to 200 micrograms per well.

The plate was placed back in the microplate reader and incubated for 5 more hours with readings taken every hour at an absorbance of 600 nm wavelength. The first reading was taken in the first minute, followed by five more readings at one-hour intervals. The experiment was repeated three times, and the mean results were analyzed.

Figure 13 Microplate Reader in working and 96 well Flat Bottom Plate with Samples

3.2.6 Addition of stress factors along with Oxamate

Fresh bacterial culture was prepared from an overnight pre-culture and incubated in a shaking water bath at 37°C. Ten wells of three rows of a 96-well microplate were used for culturing, where well 1 in first row served as a blank and well 2 (of first row) as a control using GM17 medium and pre-culture. Culture media was added to wells 3 to 10 of the first row and 2 to 10 wells of the second and third rows.

The microplate was then placed in a microplate reader with an incubation temperature of 37°C and a kinetic loop of 3 hours with an interval of 1 hour. The absorbance was set at a wavelength of 600nm with continuous shaking. The initial absorbance result was calculated in the first minute, and three further absorbance values were calculated after a one-hour interval.

After three hours of incubation, the experiment was stopped when the OD reached 0.8. The plate was then removed, and drug solutions (5 to 200ug) were added to each well of the first row except for wells 1 and 2, which served as blank and control, respectively. In second row, wells 2, 3, 4 were filled with 0.01% SDS along with the different

concentrations (100ug, 150ug, 200ug respectively) of inhibitor. Wells 5, 6, 7 of the second row were added with 2.5mM H_2O_2 along with inhibitor concentrations. Wells 8, 9, 10 were filled with 8% Ethanol with inhibitor concentrations.

In the third row, along with inhibitor concentrations, 10% DMSO was added. In wells 5, 6, 7, the stressor added with inhibitor was 10% glucose. While in 8, 9, 10 wells of the third row, HOCl 0.25% was added along with different concentrations of inhibitor.

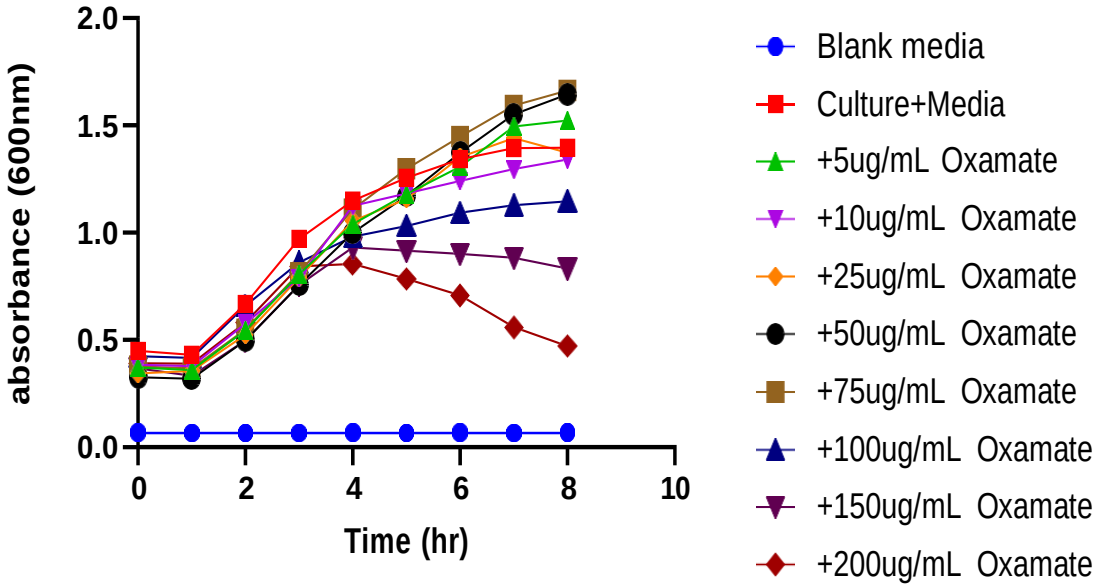
The plate was placed back in the microplate reader and incubated for 5 more hours with readings taken every hour at an absorbance of 600 nm wavelength. The first reading was taken in the first minute, followed by five more readings at one-hour intervals. The experiment was repeated three times, and the mean results were analyzed.

CHAPTER#4 Results

4.1 Effect of different concentrations of Oxamate on *E. faecalis* growth

There is no significant growth inhibition at 5, 10, 25, 50, 75 and 100 microgram per milliliter. There is significant growth inhibition at 150 and 200 microgram per milliliter.

Different concentrations of Oxamate such as 5, 10, 25, 50, 75, 100, 150 and 200 µg/mL were added to the *E. faecalis* culture after 3 hours (hrs.) of pre-incubation and later on



incubated for 5 hrs. No significant difference was observed between control and Oxamate at 5, 10, 25, 50 and 75 ug/mL. Little inhibition was shown at 100 µg/mL concentration of Oxamate (Fig.14). However, there was a prominent difference between control and Oxamate at 150 µg/mL concentration after incubation with inhibitor (Fig.14). Similarly, Oxamate at 200 µg/mL concentration showed significant difference compared to control during 4th hr. and 5th hr. of incubation (Fig.14).

Figure 14 Effect of different concentrations of Oxamate on the growth of E. faecalis.

4.2 Minimum Inhibitory Concentration of Oxamate:

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

incubation (Kowalska-Krochmal & Dudek-Wicher, 2021). The Minimum Inhibitory Concentration of Oxamate is observed as 100 ug/mL because this is the lowest dose at which a notable inhibition in bacterial growth can be observed. As a result, we compared the MIC value of Oxamate with the different six Stress variables and found combined results of inhibition.

4.3 Effect of different concentrations of Oxamate in adjunct to different stress factors on the *E. faecalis* growth

E. faecalis is known to survive harsh environments and resist various types of different stress factors (Solheim et al., 2009; Solheim et al., 2014). Therefore, we evaluated Minimum Inhibitory concentrations of Oxamate that showed significant inhibition (100 µg/mL) of the growth of *E. faecalis* in adjunct to various stress factors such as Sodium Dodecyl Sulphate (SDS, 0.01% w/v), Hydrogen Peroxide (H_2O_2), Ethanol (8% w/v) and Dimethyl Sulfoxide (DMSO, 10% w/v),10% Glucose, 0.25% HOCl.

4.3.1 Effect of different concentrations of Oxamate in adjunct to 0.01% w/v Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate (SDS) is a detergent and is used as a stress factor for the growth of *E. faecalis*. After 3 hrs. of pre-incubation, 100 µg/mL concentration of the Oxamate along with 0.01% SDS displayed significant inhibition during the 3rd, 4th, and 5th hr. of post-drug and stress incubation period. (Fig.15).

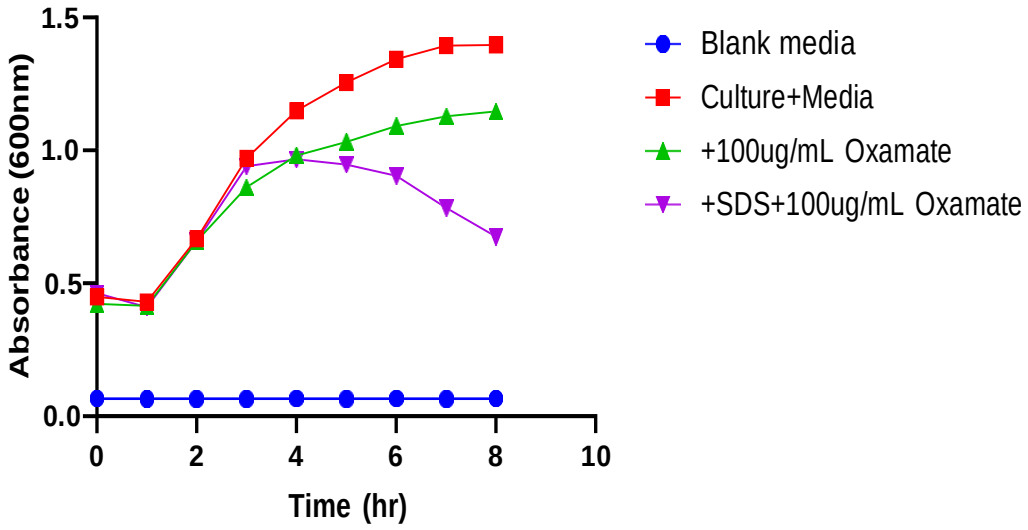


Figure 15 Effect of different concentrations of Oxamate in adjunct to 0.01% w/v Sodium Dodecyl Sulphate (SDS)

4.3.2 Effect of different concentrations of Oxamate in adjunct to 2.5 mM Hydrogen Peroxide (H_2O_2)

Similar to SDS stress, H_2O_2 was used as stress factor at a concentration of 2.5 mM to evaluate the increase in growth inhibition in adjunct to the Oxamate. Application of H_2O_2 did not significantly reduce the growth of *E. faecalis* when used in combination with different doses of the Oxamate. (Fig.16)

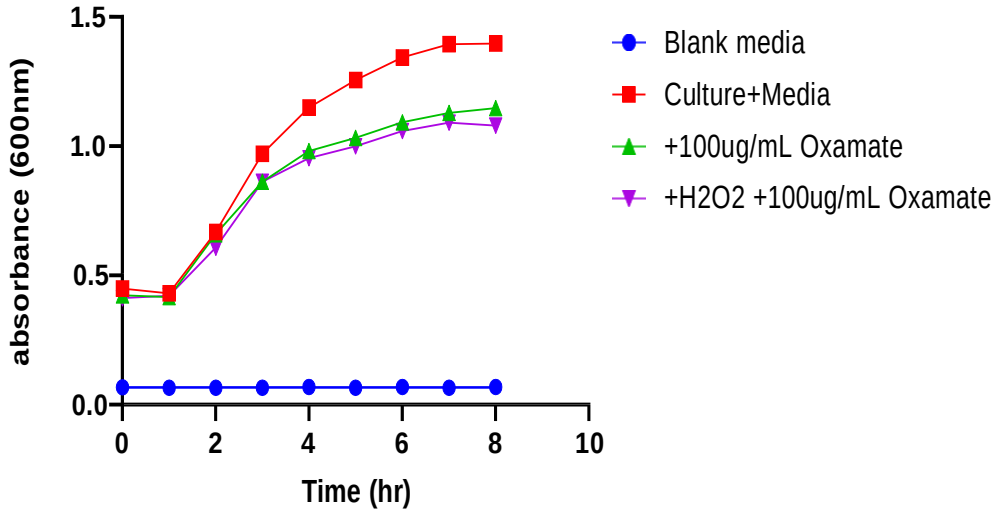


Figure 16 Effect of different concentrations of Oxamate in adjunct to the application of Hydrogen Peroxide (H2O2) stress

4.3.3 Effect of different concentrations of Oxamate in adjunct to 8% Ethanol

Application of 8% Ethanol induced stress along with 100 µg/mL concentration significantly inhibited the growth of *E. faecalis* during 2nd and 3rd hrs. of incubation after the addition of Oxamate and 8% Ethanol in comparison to the control (Fig. 17). Although, the combination of Ethanol and Oxamate appeared to reduce the growth of *E. faecalis* more than when Oxamate was used alone at these concentrations, statistically significant difference was observed.

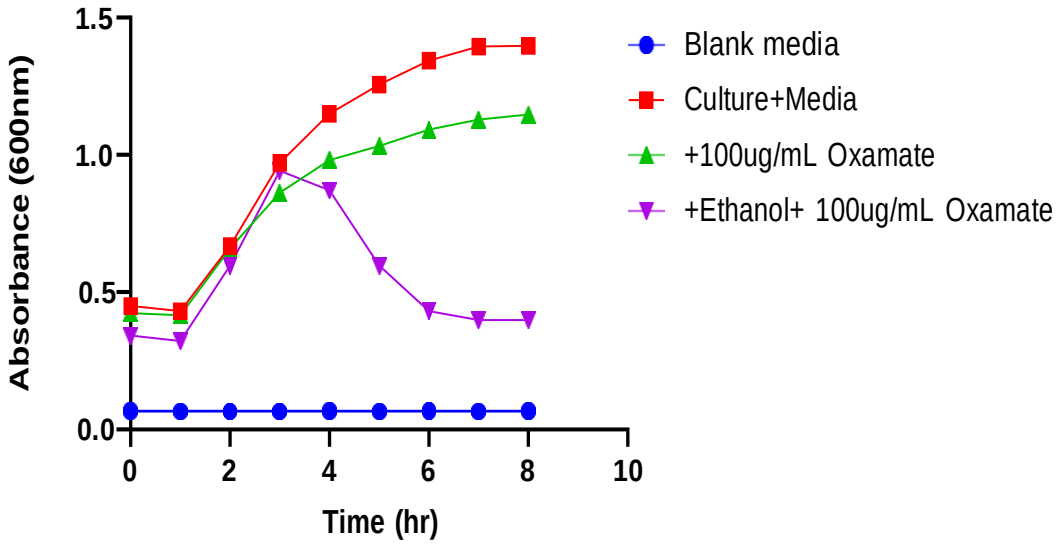


Figure 17 Effect of different concentrations of Oxamate in adjunct to the application of 8% Ethanol stress

4.3.4 Effect of different concentrations of Oxamate in adjunct to Dimethyl Sulfoxide (DMSO)

Combined application of the Oxamate at different concentrations such as 100 µg/mL with 10%DMSO did not significantly inhibit the growth of *E. faecalis* as compared to 100ug/mL of oxamate alone (Fig.18)

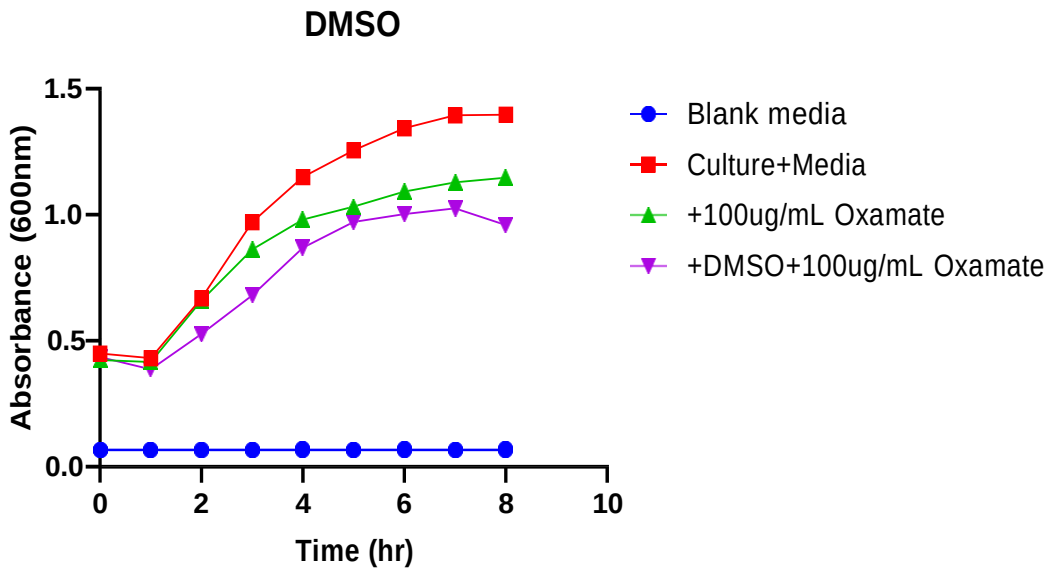


Figure 18 Effect of different concentrations of Oxamate in adjunct to the application of 10% DMSO stress

4.3.5 Effect of different concentrations of Oxamate in adjunct to 10% Glucose

Combination of 10% Glucose and 100 µg/mL concentration of Oxamate significantly inhibited the growth after the 2nd hrs. of post-incubation period compared to the control and 100ug/mL when used alone (Fig.19)

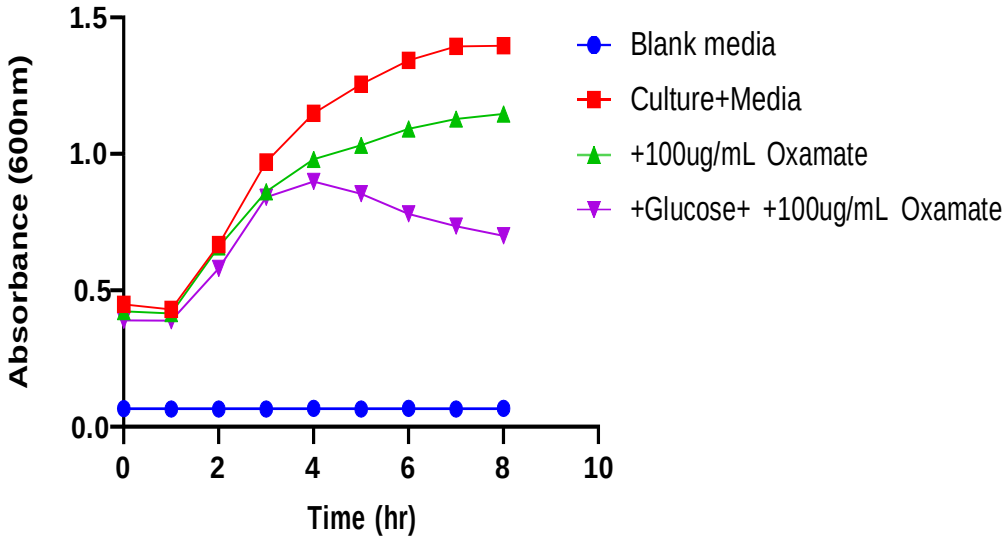


Figure 19 Effect of different concentrations of Oxamate in adjunct to the application of 10% Glucose stress

4.3.6 Effect of different concentrations of Oxamate in adjunct to HOCl

Combination of 0.25% HOCl and 100 µg/mL concentration of Oxamate significantly inhibited the growth during the 4th, and 5th hrs. of post-incubation period compared to the controls and 100ug/mL when used alone. (Fig.20)

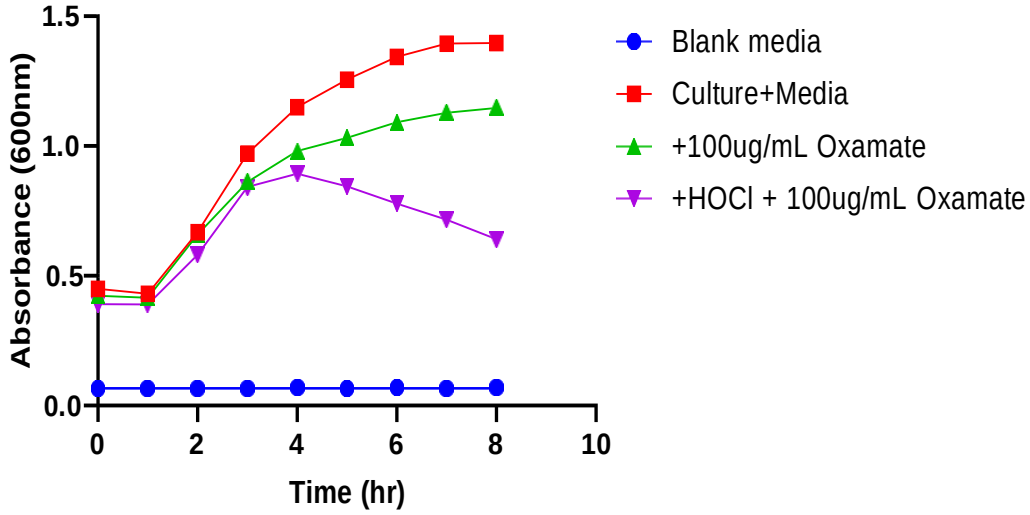


Figure 20 Effect of different concentrations of Oxamate in adjunct to the application of HOCl induced stress

4.4 Kinetic Assay for the determination of Lactate Dehydrogenase Activity by DGKC Method:

Lactate Dehydrogenase (LDH) Enzyme activity was determined after incubation without and with Oxamate different doses. The different doses of Oxamate did not show any impact on LDH activity except a slight increase in LDH activity that was seen after incubation with Oxamate 100 µg/mL. This slight increase in LDH activity could be speculated to stem from increased expression of LDH enzyme due to probably strong inhibition at this dose of Oxamate. (Fig.21)

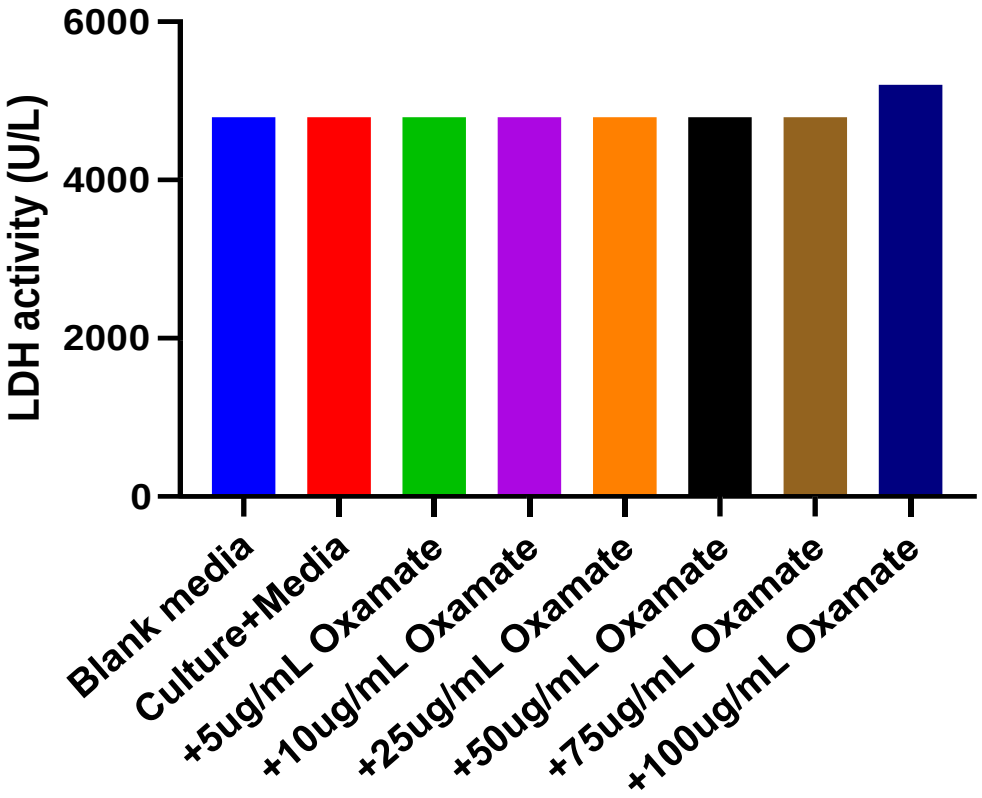


Figure 21 Determination of Lactate Dehydrogenase Activity at different concentrations of Oxamate

Discussion

Lactate Dehydrogenase enzyme is primarily used by *E. faecalis* for growth, virulence, and resistance. There are two forms of lactate dehydrogenase enzyme in this bacterium:

LDH1 and LDH2, with LDH1 playing a crucial part in how this bacterium acts. Oxamate, an oxamic acid salt, with numerous medicinal benefits as well as an inhibitor of the Ldh enzyme in cancer cells, is one of the many ligands for the inhibition of this important enzyme that have been found by in-silico approaches. We utilized one of Ligand Oxamate for evaluating its anti-bacterial activity. A preliminary step towards evaluating Oxamate on animal models and toxicological assessments for preventing Enterococcus faecalis infections was demonstrated by an in vitro analysis against the pathogen. Optimized Oxamate concentrations for antibacterial activity and calculated its MIC value. We combined Minimum Inhibitory concentrations of Oxamate100µg/ml with six stressors, including 0.01% (w/v) Sodium Dodecyl Sulfate (SDS), 2.5mM Hydrogen Peroxide (H_2O_2), 8% (w/v) Ethanol, Dimethyl Sulfoxide (10%w/v), 10% Glucose and 0.25% HOCl. Despite being given at MIC of 100 ug/mL , DMSO and H_2O_2 have similar effects and do not appreciably suppress the growth of *Enterococcus faecalis*. At MIC value of Oxamate dose of 100 ug/mL at the second and third hours of the bacterial inhibitor interaction, ethanol at a concentration of 8% exhibits considerable inhibition. After the drug's post-incubation period with 0.01% SDS, the addition of Oxamate at MIC of 100 ug/mL significantly inhibits the growth of *E. faecalis*. Additionally, glucose 10% significantly inhibits growth after two hours of drug incubation. While 0.25% significantly inhibits growth during hours four and five after drug incubation.

Conclusion

Oxamate is a drug used in several anticancer therapies worldwide. It blocks the activity of enzyme LDH rendering the reduced production of lactic acid and ultimately stops the proliferation of cancer cells. . *E. faecalis* is a lactic acid bacterium that is extremely resistant and maintains its redox balance by LDH activity as it undergoes homo

fermentation. (Brown & Wittenberger, 1971; Rana et al., 2013). Under these conditions, lactate is the primary or main end product of fermentation (M. Jönsson et al., 2009; Rana, 2012) just like cancer cells. Keeping this into account, this study attempted to use oxamate as a targeted drug to inhibit the activity of LDH to weaken and eventually causing death of the bacteria. The results of this study demonstrated for the first time, the ability of oxamate to inhibit the LDH enzyme in the bacteria proving its potential as an antibiotic. When administered with other physiological stressors, it showed remarkable results in inhibiting the growth of the bacteria proving it to be an effective treatment for reducing the LDH activity hence minimizing its resistance and making it vulnerable to different stressing conditions which otherwise showed no harm against the bacteria. In future, in-vivo studies are required to demonstrate the antibacterial activity of oxamate in detail and also for comparing it to the other antibiotics as a combined therapy.

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