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Estimation of Inhibitory Action of Postbiotics against Neonates Listeria Monocytogenes

by

Irsa kanwal

A thesis submitted in partial fulfillment for the degree of Master of Science

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I dedicated this thesis to my family members and teachers.

CERTIFICATE OF APPROVAL

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(Irsa Kanwal)

Abstract

Listeriosis is a serious bacterial infection disease caused by Listeria monocytogenes of both gram positive and gram negative bacteria. This disease most commonly affects neonatal baby, pregnant women and also affect people with weak immune system. Advancement in screening techniques and treatment methodology have important role in reducing the symptoms of listeriosis disease. Globally people are more involved in using a postbiotics products which have a significant effects on host directly and indirectly because these products produce metabolic activity due to microorganisms. Probiotics are those substances which have beneficial effects on human gut microbiota. Due to probiotics in take, human gut microbiota cannot disturb. But now the focus is shifting from viable probiotics to non-viable probiotics because postbiotics contain potential application in different sectors like pharmaceuticals and food industries. Postbiotics contain a metabolic constituents such as short chain fatty acids, acetyl alcohol, bacteriocins, fructose-6-phosphate, Cinnamyl alcohol, beta-Caryophyllene alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid and propionic acid which are identified from Bifidobacterium *aquikifir* species. These metabolic compounds contain anti-inflammatory activity, anti-diabetic activity, anti-bacterial activity, anti-cancer activity, anti-oxidant activity and also significant role in pharmacological activity. Computer aided drug designing is a modern technique currently mostly used to design a drug for different purposes. So the virtual screenings of these compounds were carried out against drug targeted proteins that are Internalin A, Internalin B and Listeriolysin O through CB dock online tool. Caryophyllene oxide was selected as a lead compound by performing all screening filter represents Caryophyllene as a lead compound. Gentamicin is used as a reference drug for comparison. After a detail analysis and comparison Caryophyllene oxide is much more active than gentamicin reference drug. All the interaction and visualization studies were performed by using Pymol software and Ligplot plus. Finally as a result of current Insilco study, I have discovered Caryophyllene oxide as a most potential antibacterial compound which might be a drug candidate to treat listeria neonatal infection.

However further research study is necessary to investigate their potential medicinal use.

Keywords: listeriosis, probiotics, postbiotics, metabolic compounds, Bifidobacterium *aquikifir*, Computer aided drug designing, CB dock, Pymol, Ligplot, Caryophyllene oxide & gentamicin.

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Abbreviations

B. *aquikefiri*	Bifidobacterium *aquikefiri*
BBB	Blood Brain Barrier
CNS	Central Nervous System
CADD	Computer Aided Drug Designing
CDCF	Cholesterol Dependent Cytolysin Family
FDA	Food Drug Authority
MMSE	Mini Mental State Examination
MRTD	Maximum rate tolerated dose
PDB	Protein Data Bank
VDss	Volume of Distribution
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background

Infections of the respiratory and gastrointestinal tract remains main public health issues, particularly among children under the age of five. Children under the age of five are more vulnerable to infections, which are considered to be produced by a complex network of modulators including immature immune response and organ function [1]. However, due to uncommon instances of probiotic-related diseases such as bacteremia, necrotizing enterocolitis, pneumonia, and meningitis, a major segment of the scientific community does not favor probiotic therapies in young children [2] [3].

The United Nations Sustainable Development Goals make protecting children's lives and improving their health a key priority [3]. The great majority of newborn deaths occurs in the first month of life, and is caused by premature birth, delivery problems, and infectious diseases [4]. Diarrhea is a leading cause of mortality in children under the age of five years and having considering among infectious diseases [5]. Diarrhea common causes are due to certain bacteria contain, diarrhoeagenic Escherichia coli, Shigella species, Salmonella species, and Campylobacter and Yersinia enterocolitica. Besides E. coli rotavirus is the other most common etiological agent of moderate-to-severe diarrhea in low-income countries.

Fortunately, most of these reasons may be avoided and treated, but doing so will demand more global awareness, greater access to high-quality treatment, and affordable dietary changes. Low birth weight, heredity, and food are some of the variables that raise the chance of contracting an infectious disease. The growth of a healthy gut microbiota is vital for the development of the immune system and may minimize the risk of infectious illnesses in infants and children [6]. Diet is one approach to shape and modify the gut microbiome. Although breast milk is the greatest source of food for developing a diverse and balanced microbiota composition, it is not always available or adequate for the newborn. As a result, infant formulae are made and constantly modified in order to mimic the nutritious makeup of breast milk. Various preparations and interventions, such as probiotic supplementation, have been investigated in order to improve the composition of infant formulae throughout the last few decades [7].

Probiotics are live microorganisms that provide a health benefit to the host when given in sufficient concentrations. Prebiotics"and probiotics have received a lot of attention for their ability to improve intestinal health. Postbiotics have recently emerged as another class of helpful compounds that can help you to boost your health [8]. They have been associated with several other health benefits for the gut, immune system, and a number of other aspects of health. Probiotics when consumed in adequate quantities, provide health benefits to the host. The rationale for using probiotics to fight infections is that probiotics demonstrate a good ability to bind to intestinal mucus and compete with specific pathogens for the same adhesion sights. As a result, they prevent pathogen adhesion making pathogen propagation difficult [9]. Surface proteins of probiotic bacteria enhance the connection of probiotics to intestinal mucus or epithelial cells, which can limit pathogen attachment through competitive exclusion. Proteins, glycoproteins, lipoproteins, lipoteichoic acids, lipopolysaccharides, adhesions, and flagellins are examples of bacterial surface components [10]. As a result, these qualities encourage the addition of probiotics to newborn formulae. However, there is presently insufficient data to support the use of probiotic-supplemented formulas on a regular basis [11-15]. Probiotics may be a valuable new alternative antibacterial therapy when a

child is already infected with a disease, in addition to providing potential infection prevention. The only treatment option for bacterial diarrhea is antibiotic medication, which is typically not suggested. In addition, several pathogenic strains have acquired antibiotic resistance [16]. There is growing worry that certain probiotics may collect antibiotic-resistant genes in their genomes, which might be transmitted to other potentially pathogenic bacteria, according to the study [16,17]. Furthermore, due to rare case reports of probiotic side effects, many physicians remain skeptical about their usage in pediatric therapy [17]. A growing body of evidence shows that many probiotic strains might potentially improve host health even when they are not living [18]. For instance, it has been demonstrated that inactivated probiotics might attach to intestinal mucus better than living bacteria depending on the inactivation process [19].

Furthermore, some probiotic strains have been reported to express putative virulence factors, enhancing their tendency to adhere, invade, and cause cytotoxic effects [20]. Another issue is the possibility of antibiotic resistance genes being transferred to pathogenic bacteria in the gut [21, 22]. Current "research is looking into the use of fermented infant formulae containing inactivated probiotics and their metabolic products, as well as their role in infection prevention" [22, 23] [24]. Postbiotics, paraprobiotics, metabiotics, proteobiotics, pharma, pharmabiotics and ghost probiotics are novel terminologies that have recently developed [21, 26]. Interestingly, evidence from the literature suggests that by using the term postbiotics, one refers to either inactivated probiotic strains or their metabolic products or both. Currently, a definition is under development although postbiotics have been tentatively characterized as bioactive substances derived from food-grade microbes as a result of a fermentation process that promotes health and well-being [27]. Postbiotics is the newest member of the biotic family, referring to bioactive compounds generated by food-grade bacteria following the fermentation process. Postbiotics contain microbial cells, cell constituents, metabolites and bioactive compounds. Many researches utilize the term postbiotics. Some researches mention the use of parabiotics known as inactive microbial cells and fermented infant formulae (FIFs), which fall within the category of postbiotics.

Postbiotics are"inactive microorganisms, cell structures, and metabolites created after bacterial lysis or released during fermentation. Most studies use fermented culture medium that has been heated or filtered after the microbe has grown to create Postbiotics. This process produces a liquid known as cell-free supernatant, also known as spent culture supernatant or cell-free spent medium. Instead of a single pure substance, this suggests the presence of a mixture of bioactive compounds [28]. Postbiotics can also be obtained by inactivating probiotics with heat, filtration, sonication, centrifugation, and UV radiation, among other methods. Bacterial lysis can occur in this circumstance, releasing a variety of substances such as DNA, enzymes, lipoteichoic acids, and other intracellular metabolites that might serve as potential biomarkers postbiotics [29].

The clinical effects of postbiotics for preventing and treating common infectious diseases in children were studied in a systematic review published in 2020 [29]. Given that there is limited research on postbiotics, coupled with their potential beneficial effects. On the basis of existing knowledge, this study explores and presents probable preventative mechanisms and uses of postbiotics against pediatric infectious diseases. Members of the genus Bifidobacterium are significant because of their supposed health-promoting benefits in humans throughout their lifespan. Their existence in the human gastrointestinal tract is frequently associated with health advantages including the production of metabolites such as short-chain fatty acids and vitamins, the development of the immune system, and the prevention of gut problems [30]. Gut dysbiosis is now accepted to refer to changes in the quantitative and qualitative composition of microbiota, and that these changes can lead to altered host microbial interaction, which can contribute to a disease state, often with inflammation, and that this is linked to the development of many non-communicable human diseases [30-31]. Furthermore, compositional alterations of the gastrointestinal tract microbiota have been linked to certain gastrointestinal diseases such as Clostridium difficile–associated diarrhea in adults and children inflammatory bowel disease and necrotizing enterocolitis [32]. Listeria monocytogenes, a facultative rod-shaped Gram positive bacterium, causes listeriosis, a severe and sometimes fatal infection spread mostly

through contaminated food. Listeria monocytogenes is an opportunistic human and animal foodborne pathogen. L. monocytogenes has the highest case-fatality rate of any foodborne pathogen in developed countries. L. monocytogenes preferentially"infects pregnant women, newborns, those with impaired immune systems, and the elderly. Due to the intrinsic immune suppression of pregnancy, pregnant women are at around 18 times the risk of infection as the general population. While maternal infections are frequently benign, newborn illnesses can be serious and even fatal [33]. Vertical transmission of L. monocytogenes can result in neonatal listeriosis, from mother to fetus by inhalation of contaminated amniotic fluid, trans-placental transmission from the maternal circulation, or ascending colonization during delivery. The gestational age at which infection develops influences clinical outcomes. Listeriosis is most frequent in the third trimester of pregnancy (from 28 weeks) and is seldom deadly in the mother, especially if no other medical issues are present. Later infection, especially in the third trimester, is usually associated with better fetal outcomes than earlier infection. If the pathogen is transmitted to the fetus, it might result in miscarriage, premature birth, or stillbirth. A recent research of 107 instances of pregnancy-related listeriosis in France found that infection was passed from mother to fetus in 96% of cases, and severe fetal or neonatal problems were identified in 83% of babies born"to infected mothers [34]. Neonatal listeriosis typically manifests as bacteremia, respiratory distress, meningitis, and, less commonly, pneumonia within the first 24 to 72 hours of life. The most prevalent cause of late-onset listeriosis, which manifests in neonates aged one to four weeks, is meningitis. Approximately half of all L. Monocytogenes-infected newborns do not appear to be immunocompromised. The overall case fatality rate for newborn listeriosis is 50%, with 40% of surviving neonates showing major neurological and developmental abnormalities. In immunocompetent youngsters, listeriosis is most likely to present as an influenza-like illness or, if the infection is severe, gastroenteritis. In immunocompromised children, however, infection can manifest as a number of clinical syndromes, the most prevalent of which are meningitis and bacteremia [35]. Recent breakthroughs in bifidobacterial studies demonstrate that bifidobacterial strains coevolved with their hosts and that many

physiological characteristics might be dependent on residential origin [36-37]. In this context, it has been established that specific bifidobacterial species live naturally within the human host.

However, the causes of bifido bacterial species persistence in human's gastrointestinal tracts during the course of their lives, their adaptability to and survival in the hostile environment of the gastrointestinal system, and their effects on human health are still unknown.

An investigating their functional characteristics as members of the human gut microbiota and human niche-specific adaptation is crucial.

1.2 Hypothesis

Postbiotics are secreted metabolites or by product of probiotic strains. Probiotics are responsible for multiple important health boosting function. If postbiotics are used insted of living probiotics, they could eliminate health risks associated with the use of living probiotics.

1.3 Problem Statement

The live probiotics affect the various host specific factor in the gastrointestinal tract. There are some health risk associated with live probiotics in neonates that can cause disease and also disturb the environment in GIT.

1.4 Aim

To identify bioactive compounds of probiotics for their potential to decrease disease estiblishment by L. Monocytogens.

1.5 Objectives

The objectives of this study are:

- To screen bioactive compounds of B. *aquikefiri* as postbiotics metabolits.
- To check effectiveness of selected metabolites as antiadhesive molecule against Listeria monocytogenes.

Chapter 2

Literature Review

2.1 Probiotics

The gut microbiota is the most complicated ecosystem in nature because it contains large bacterial populations in the intestine and colon, with around 1011–1012 microorganisms/gram of intestinal content, the majority of which are anaerobes (95 percent of total organisms) [38]. The first studies on the composition of intestinal microbiota were based on microscopic observation and culture-based methods, and showed as predominant cultivable species Bacteroides spp., Eubacteria spp., Bifidobacterium spp., Peptostreptoccocus spp., Fusobacterium spp., Ruminococcus spp., Clostridium spp. and Lactobacillus spp [39]. Fermented dairyproducts are the key sources of probiotic bacterial strains. Lactic acid bacteria including probiotic Lactobacillus spp. have been widely used as starter culture in several fermented dairy products. Bifidobacterium along with Lactobacillus seems to be the most promising microbial genera in health-promoting dairy foods formulations [40]. The microbiota is an assemblage of microorganisms that inhabit the human body, their genomes and metabolites, as well as the environment in which they exist. Microorganisms that are part of the micro biome can be isolated from all areas in constant contact with the external environment (e.g., the skin, upper respiratory tract, or urogenital tract) [41] [42]. Functional attributes of these bacteria

contribute directly or indirectly to several health benefits including the protection against pathogenic microbes, hypertension, inflammation, diabetes, oxidative stress, etc. These microbes are also involved microbiome modulation, immune modulation, and anti-cholesterol emic activity [43]. Systematic diagram of probiotics was shown in Figure 2.1.

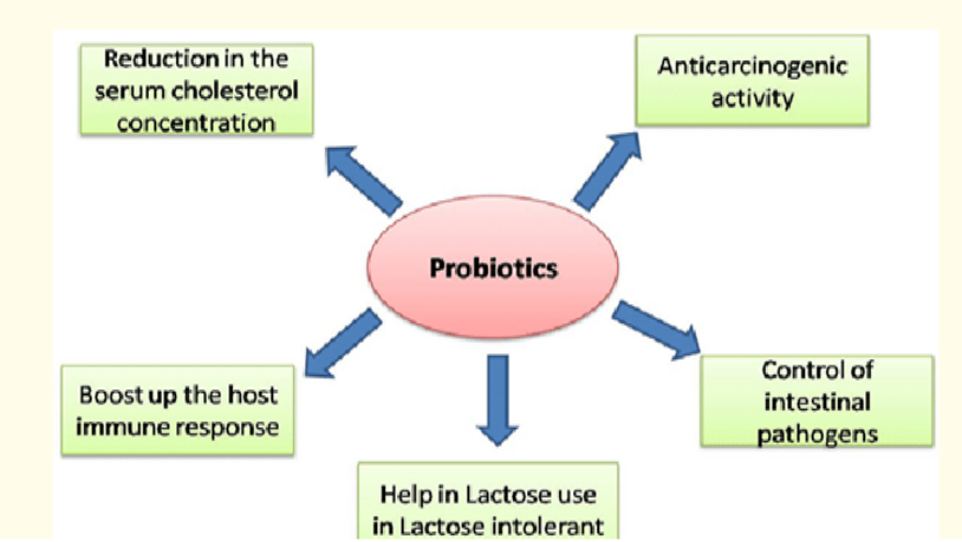


FIGURE 2.1: Systematic diagram of probiotics [44].

2.2 Bifidobacterium

Bifidobacterium are Gram-stain-positive, non-motile, nonspore forming bacteria that are usually associated with the gut microbiota of humans and animals [45]. They are generally obligated anaerobic but some species can also grow aerobically [46]. A number of bacterial genera primarily belonging to Enterococcus, Enterobacter, Escherichia, Bifidobacterium, and Lactobacillus colonize the human gut. Probiotics bacterial strains with genera and species were listed in Table 2.1. Among these bacteria, Bifidobacterium is one of the most abundant bacteria of healthy breast-fed infants. This bacterium has demonstrated notable physiological and genetic features along with the adhesion ability to epithelial cells and metabolism of host-derived glycans. B. bifidum and B. breve are the most frequently shared gut colonizing species between mothers and their corresponding children [47].

TABLE 2.1: Following table shows us probiotics bacterial strains with genera and species.

S.no	Genera	Species
1	Bifidobacterium	*B.bifidum*
		B.breve
		B.aquikefir
2	Lactobacillus	*L.crispatus*
		L.jensenii
		L.gasseri
3	Enterococcus	*E.faecalis*
		E.faecium
		E.avium
4	Escherichia	*E.albetii*
		E.fergusonii
		E. vulneris.
5	Saccharomyces	*S.bayanus*
		S.paradoxus
		S.uvarum

2.2.1 Bifidobacterium *aquikefir*

Bifidobacterium, strain B.aquikefiri, was isolated from a household water fermentation process B. aquikefiri are Gram-stain-positive, non-filamentous, nonmotile, non-spore-forming, catalase-negative and oxidase negative. They form short rods 0.5–1.0 μm thick and 1–2 μm long without bifurcations [48]. Bifidobacterium strains recently reported key functions were given in the following table 2.2.

2.3 Neonatal Listeria infection

Listeriosis is a bacterial infection caused by Listeria monocytogenes (L. monocytogenes). The bacterium that frequently causes infection is L. monocytogenes.

TABLE 2.2: Recently reported key function of different Bifidobacterium strains.

S.No	Bacterial Strains	Functions
1	Bifidobacterium *bifidum*	anti-inflammatory role by modulating miRNA-associated Tight Junction-related Protein (TJP) and NF-B regulation and, restoring dysbiosis.
2	Bifidobacterium *animilas*	Reduced infant colic (Modulation of gut microbiota structure and function)
3	Bifidobacterium *longum*	Modulated neural responses during social stress
4	Bifidobacterium *breve*	Improving memory functions
5	Bifidobacterium *aquikefiri*	Growth occurs under anaerobic, micro aerobic and aerobic conditions, from pH 4.0 to 8.0, and at 4–37 °C.

Certain inhabitants, particularly high-risk individuals including the elderly, immunocompromised patients, and pregnant women. It can, however, affect individuals who do not possess these risk factors. Being a member of L. monocytogenes is common in nature. It is found in the faeces of many mammals and is a common food source. Humans obtain it primarily through intake [49]. Listeria infection is primarily transmitted through the placenta, which is a critical illness associated with a high mortality rate. The necessary dietary guidance for pregnant women can reduce the incidence rate of pregnancy-related listeriosis. Listeriosis is the most frequent foodborne illness, and it has been linked to contaminated food in sporadic cases [50]. Listeria monocytogenes is a gram-positive bacillus and faecal microorganism. L. monocytogenes was the final intracellular bacterium. L.monocytogen can survive at temperature ranging from 4-37 °C. It was first reported in 1926 by Murray et al. While they were looking into an outbreak of illness in laboratory rabbits and guinea pigs [51]. According to the World Health Organization (WHO), the onset of Listeria during pregnancy accounted for nearly 43% of total cases, and 14% occurred in late pregnancies [52]. In 1929, the first incidence of

human listeriosis was reported. A few years later, in the 1980s, there was a rise in the number of reported listeriosis cases in numerous countries, which led to the disease being recognized as a foodborne illness [53]. L. monocytogenes is usually regarded as a Bacterium in nature because it is found in the faeces of many humans. The principal route of bacterial transmission is thought to be mammals and is a common food source. Through the intake of unsafe food such as meat, vegetables, sea foods, and unpasteurized milk etc. The incubation period of listeriosis is variable and long period duration up to 1-70 days. C-section rates are one of the health insurance indexes. C-section rates, according to the World Health Organization (WHO), it was reported as 15% in 1985. According to the reports WHO estimates that this rate has significantly increased globally in 2021[54]. C-section is only suggested when the mother's or fetus's life is in danger. However, this approach is now being used to reduce labour pain. People commonly believe that caesarean birth is less painful, safer, and healthier than vaginal birth. In reality, more than half of women choose to have a C-section [55]. Listeriosis is a

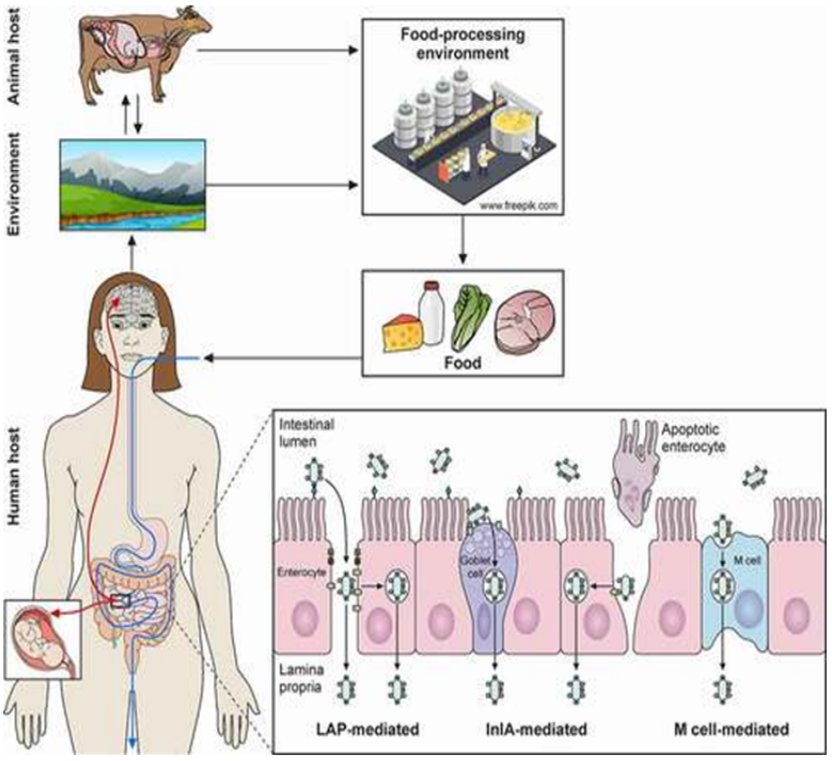


FIGURE 2.2: Listeriosis diseases mechanism of actions [50].

rare infection, although it is approximately 20 times more likely among pregnant women than in the general population [56]. Listeria disease mechanism of action was shown in above figure 2.2. Pregnant women's calculations 27% of all listerial infections cause minor illness in women, but Listeriosis is a rare infection that can be detrimental to the fetus, causing serious disease or death in some circumstances. Pregnant women can lower their risk of listerial infection by following the Centers for Disease Control and Prevention's dietary guidelines (CDC) According to estimates, 8.6 cases of listeriosis occur in newborns for every 100,000 live births [57]. In pregnancy, the incidence of listeriosis is 12 per 100,000, compared to 0.7 per 100,000 in the general population [58].

2.3.1 Incidence and Mortality rate

Recently, published data for incidence index in pregnancy- related listeriosis accounts for 11% of all listeriosis cases in Italy [59], 16% in Spain and 17.7% in France [60]. In France, the index declined from 60 to 5 cases in every 100,000 live births between 1984 and 2011, reducing by more than 12 times [61]. Moreover, 41.1–52% of listeriosis was associated with pregnancies in China, highlighting the nationwide pressure of this disease [62].

The findings of Gohar et al [63], showed a 13.6% prevalence from Pakistan, Hosseini et al showed 19.04% from Iran, and Yakubu et al showed 22.4% from Nigeria [64]. Mortality rates of listeriosis disease in Pakistan and globally shown in table 2.3.

Table 2.3: Mortality rate of listeriosis disease in Pakistan and worldwide.

S.no	Countries	Years	Mortality Rate %
1	Pakistan	2010-2020	13.6%
2	USA	2009-2011	17.6%
3	China	1964-2010	26%
4	Denmark	1994-2003	21%
5	Spain	2011	16%
6	Italy	2013	11%
7	Nigeria	2033-2007	22.4%

2.4 Gut flora in neonates with Surgical and non-surgical condition

While the gut microbiota of extremely preterm non-surgical infants has been well studied using culture-independent genomic approaches, there is very limited information on gut microbiota of term infants with CGISCs .

The studies that evaluated gut flora in neonates with surgical conditions in the past were based on the conventional culture-dependent techniques. However, a growing body of evidence in the recent decade has shown the importance of culture-independent, genomic approaches in understanding the role of the human microbiota in health and disease. Hence, we conducted this prospective study to investigate the gut microbiota in term neonates with CGISCs using culture-independent techniques [65].

2.5 Microbial colonization

Microbial colonization in early life is crucial for infant health and may affect health status in later life. Substantial effort has been devoted into studying the development of the gut microbiota during infancy .

The gut microbiota expands substantially in variety and stability throughout the first year of life, and reportedly reaches an adult-like configuration in the subsequent years [66]. Many studies have shown that early events such as birth mode, newborn feeding type, presence of older siblings, and maternal and infant antibiotic usage all influence the development and composition of gut microbiota throughout infancy. Following weaning, dietary patterns have a significant influence on the childhood gut microbiota. Factor affecting microbial colonization of the developing human represent in figure 2.3. However, we still have a gap in knowledge of the roles played by early events and lifestyle in the development of the gut microbiota during childhood [67].

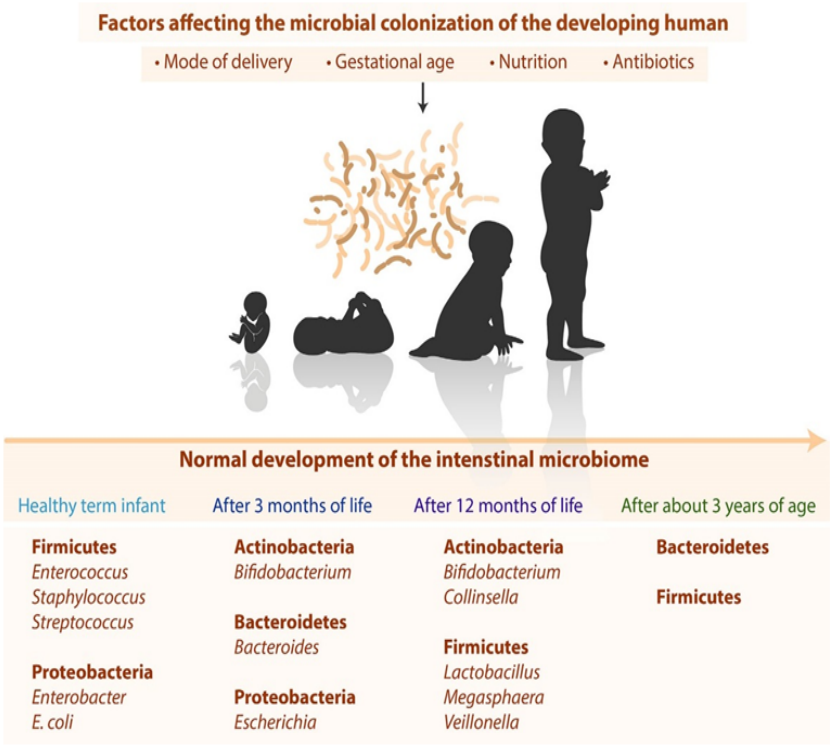


FIGURE 2.3: Factors affecting microbial colonization [67].

2.6 Symptoms Treatment and Diagnosis of Listeriosis Neonatal Disease

Pregnant women's infections can be asymptomatic or have an underlying bacterial infection that appears as a nonspecific flu-like illness. Listeriosis neonatal disease is mostly treated with antibiotics like ampicillin and gentamicin. Ampicillin, an extended-spectrum penicillin, is effective against both Gram positive and Gram negative bacteria .

Ampicillin is also used in conjunction with other antibiotics such as (aminoglycosides, β-lactamase inhibitor) to increase efficacy, antimicrobial coverage, and growth inhibition against drug resistance [69].

Gentamicin has been shown to be effective against a wide range of infections, including Gram-negative organisms and methicillin-resistant staphylococci [70]. Listeriosis neonatal disease should be diagnosis through polymerase chain reaction (PCR) or culture sensitivity testing.

2.6.1 Selection of Drugs Against Listeria Neonatal Disease

There are several drugs or medications which can be mostly used to treat listeria neonatal disease. Specifically two drugs such as ampicillin and gentamicin is mostly recommended for the treatment of this disease, these drugs can prevent or slow down the mechanism of action against disease [71]. These drugs produce their therapeutic activity through inhibition of Internalin A, Internalin B and Listeriolysin O. Reference drugs with its mechanisms of action and side effects was shown in Table 2.4.

TABLE 2.4: Reference drugs with its mechanisms of action and side effects.

S.No	Name of Drugs	Mechanism of action	Side Effects
1	Ampicilin	Ampicillin works by binding to penicillin-binding proteins (PBPs), inhibiting the formation of cell wall peptidoglycans, and using inhibitors to stop the activity of autolytic enzymes.	Hives, rash, diarrhea ,skin sensitization, fever, redness and peeling of skin.
2	Gentamicin	It works by attaching to the 30S ribosome and preventing the synthesis of bacterial proteins.	Agitation, back pain, abdominal pain, blurred vision, numbness, burning and blood in urine.

2.7 Targeted proteins

There are three different types of proteins which are used as a targeted protein for our research studies such as Internalin A, Internalin B and Listeriolysin O.

2.7.1 Internalin A

Internalin A (InlA), a protein necessary for Listeria monocytogenes pathogenicity, is encoded by the InlA gene, which is only present in pathogenic strains of this genus. Detecting a strain is one of the greatest ways to detect and confirm its pathogenicity. Internalin A (InlA) has a surface area of 80 kilo Dalton. Listeria enters cells via this protein and it is complicated protein. The InlA gene encodes the main virulence factor protein. Only applicable to L. monocytogenes and not to other groups for the sake of species or other species. They facilitates the adhesion of Listeria, as well as hepatocyte, epithelial, and lymphocyte invasion the endothelial cells Adhesion and invasion of bacteria. Human intestine epithelial cells are also involved in the process particular interaction with the E-cadherin receptor on host cells [72]. Mechanism of action of Internalin A was shown in Figure 2.4. Transcytosis mediated by InlA, which facilitates crossing of intestinal barriers. Internalin A is known to control phases in L. monocytogenes Faction. Internalin A binds to the surface of Listeria and promotes bacterial internalization in mammalian cells by interacting with certain host surface receptors.

2.7.2 Internalin B

Bacterial surface proteins called Internalin B (InlB) bind to the E-cadherin and Met receptors on host cells, allowing bacteria to be taken in by non-phagocytic eukaryotic cells [73]. Internalin B stimulates its receptor, c-Met, and promotes the endocytosis of junctional components such as E-cadherin. This protein promotes L.monocytogen internalization into non-phagocytic cells, where it can develop as a facultative intracellular in the cytosol. Pathogens spread to nearby cells via actin-based mortality [74]. Another surface protein is InlB, which binds to c-Met, a receptor tyrosine kinase (RTK) and the native receptor for hepatocyte growth factor (HGF), which is involved in invasion [75]. The invasion of various mammalian cell types is promoted by InlB. Mechanism of action of Internalin B was shown in Figure 2.5.

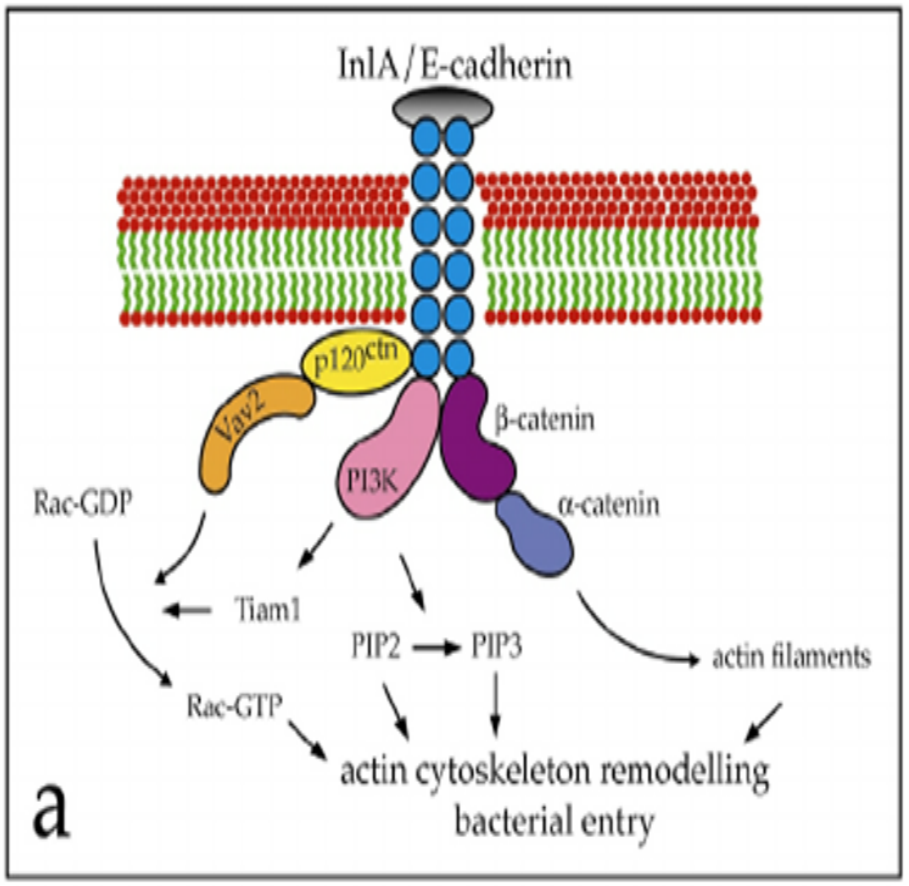


FIGURE 2.4: Internalin A protein activation mechanisms [72].

2.7.3 Listeriolysin O

Listeriolysin O (LLO) is a cholesterol-dependent cytolysin that allows Listeria monocytogenes to evade phagosomes and proliferate within the host. LLO is a powerful protein that enables Listeria to elicit a variety of cellular responses. Uncontrolled expression of LLO can cause organelle perforation and the rupture of the host plasma membrane from within the cell, resulting in cell death and the destruction of L. monocytogenes' intracellular niche, exposing the bacterium to the host immune system [77]. Listeriolysin O is the only cytolysin produced by an intracellular pathogen. As a result, LLO is best at a neutral acidic pH and can be denatured to decrease its cytolytic action at neutral pH. Mechanism of action of LLO was shown in Figure 2.5. LLO has distinct qualities that limit its cytotoxicity. However, there is a link between pore formation and the ability to control LLO activity via MAP kinase pathways [78].

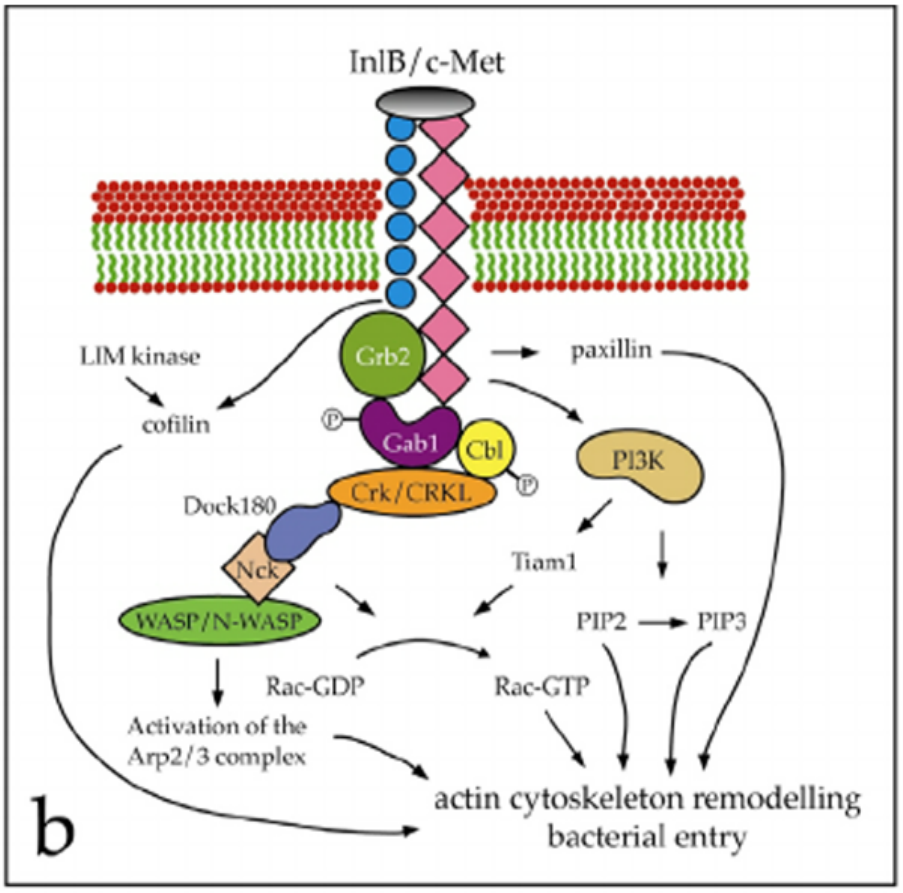


FIGURE 2.5: Mechanism of action of Internalin B [76].

2.8 Postbiotics

Postbiotics are bioactive substances produced by probiotic bacteria after they consume prebiotics (fiber). Postbiotics, the newest member of the biotics family, are bioactive compounds produced by food-grade bacteria during the fermentation process [80]. Microbial cells, cell constituents, and metabolites are examples of postbiotics. The number of articles presenting research on probiotics and prebiotics has increased dramatically over the last 40 years, indicating a rising interest in dietary strategies to control the gut microbiota. Reports on post biotic products have been emerging during the past 5 years [81]. Many of these research utilize the word postbiotics, while others mention applications such as para-probiotics, non-viable microbial cells and fermented infant formulas (FIFs) that fulfill the criteria"of postbiotics. These terms, and their synonyms started to appear after 1986 and the use of these terms is increasing [82].

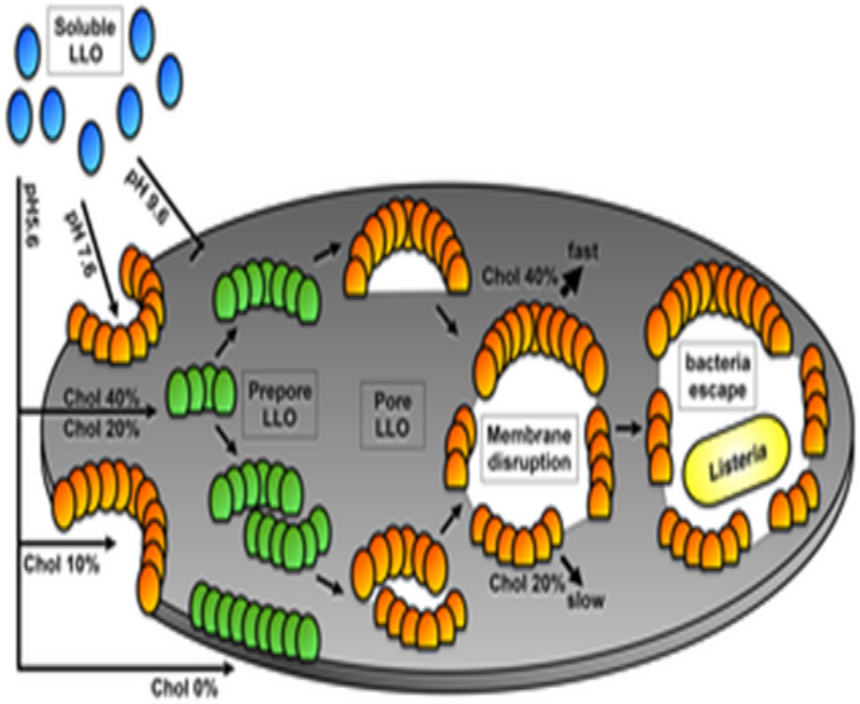


FIGURE 2.6: Schematic representation of LLO membrane disruptive mechanism of action [79].

2.9 Active Metabolites of Postbiotics as Inhibitor

Active metabolites have been described as a compound that causes a specific biological reaction in human as well as in animals. So for this purpose we have select ten different active metabolites which can inhibit the activity or over expression of proteins [83]. The active metabolites are enlisted below.

2.9.1 Short chain fatty acid

Short chain fatty acids (SCFAs), significant metabolites produced by bacterial fermentation of fiber and resistant starch in the colon, are thought to play an important role in neuro immune endocrine regulation. The main metabolic products of anaerobic bacterial fermentation in the intestine are short-chain fatty acids (SCFAs) [84]. SCFAs influence a variety of functions in the gastrointestinal (GI) tract, as well as other tissues such as adipose and immunological tissues. SCFAs

are important in the regulation of gut health. SCFAs are largely absorbed from the colon and used as a primary substrate for energy production by enterocytes. SCFAs can also prevent pathogen invasion and colonization by reducing gut pH. SCFAs can boost the immune response by promoting the synthesis of cytokines (e.g., TNF-α, IL-2, IL-6, and IL-10) in the host's immune cells [85].

2.9.2 Acetyl phosphate

Acetyl phosphate, an AckA-Pta pathway intermediate, functions as a global signal in E.coli. It is unclear whether acetyl phosphate acts directly as a phospho donor or indirectly. High energy, acid/base labile acetyl-P is an intermediate. Pta-AckA pathway, which is reversible. This pathway involves the interchanging of acetate with Coenzyme A (HS-CoA, ATP, and acetyl Coenzyme A CoA) [86].

2.9.3 Bacteriocin

Bacteriocins are a diverse class of bioactive microorganisms. Ribosomally produced peptides or proteins have antimicrobial activity against other bacteria [87]. Bacteriocins are included peptides or proteins with varying biochemical features, molecular weight, activity spectrum, location, method of action and amino acid sequence. They appear to have antibacterial activity against the same bacterial strain that has been developed against strains of these or closely related species. Bacteriocin production is controlled by genes situated in plasmid or chromosomal DNA that, in turn, the genetic factors of producer resistance are included [88].

2.9.4 Fructose 6-phosphate phosphoketolase

Only aerobic strains of Acetobacter xylinum and anaerobic bacteria of the genus Bifidobacterium exhibit the unusual bacterial enzyme fructose-6-phosphate phosphoketolase. The distinctive main enzyme of the "Bifid-shunt" is termed F6PPK

[91]. This enzyme appears to be lacking in anaerobic Gram positive bacteria. In order to test the F6PPK activity, cellular extracts were used. The gram-positive and rod-shaped bacteria's most obvious and trustworthy characteristic in the Bifidobacterium genus. Fructose-6-phosphate phosphoketolase enzyme breaks down carbon-carbon bonds and is a member of the lyase family, specifically the aldehyde-lyases.

2.9.5 Cinnamyl Alcohol

Isoforms of cinnamonyl alcohol dehydrogenase (CAD, EC 1.1.1.195) were free of periderm (including suberized and lignified cell layers). Two isoforms (CAD 1P and CAD 2P) were initially defined, and the primary isoform, CAD 1P and CAD 2P has been found. The minor form, CAD 1P, was a 34,000 molecular weight monomer that was incompatible with either aromatic or aliphatic ADH activity. CAD 2p is a dimer comprised of two opposing subunits with a natural molecular weight of around 84,000. Cinnamyl alcohol, also known as styrene [92], is an organic molecule found esterified in storax, Peru balsam, and cinnamon leaves. Cinnamyl alcohol occurs in trace levels in nature, therefore its industrial demand is mainly fulfilled through chemical synthesis.

2.9.6 Beta-Caryophyllene Alcohol

Beta-Caryophyllene alcohol is a fragrance ingredient found in cosmetics, fine scents, shampoos, toilet soaps, and other toiletries, as well as non-cosmetic items including home cleansers and detergents. Global usage is estimated to be around 0.1 metric tons per year. β-Caryophyllene (BCP), a CB2 receptor agonist, was used to study the role of CB2 receptors in controlling alcohol consumption and ethanol-induced conditioning. Evidence has accumulated since the discovery of the ECS that alcohol interacts with the ECS and that the cannabinoid receptors CB1 and CB2 play an essential and extensive role in the formation of alcohol dependency, suggesting that these receptors may be viable as therapeutic targets [93].

2.9.7 Caryophyllene oxide

Caryophyllene and Caryophyllene oxide are natural sesquiterpenoids that are useful in chemical and medical chemistry. Because of their unique structure, sesquiterpenoids can be thought of as a universal platform for chemical synthesis. Structures of various types, including biologically active compounds. Among them, the Caryophyllene group is the smallest but the most widespread in nature. Caryophyllene 1 and Caryophyllene oxide 2 are the main representatives of this group [94].

2.9.8 Caffeic acid

Caffeic acid (CA) is a phenolic chemical found in all plant species that is manufactured and present in foods such as coffee, alcohol, and tea, as well as popular drugs such as propolis. This phenolic compound acid and its derivatives have anticarcinogenic, anti-inflammatory, and antioxidant properties. A chemical against hepatocellular carcinoma (HCC), a serious kind of cancer, is being studied. In vast numbers, highly aggressive, and causes significant mortality ratio throughout worldwide [95].

2.9.9 Gallic acid

Gallic acid is the main simple polyphenol present in quinic acid ester. While the amount of theogallin is reduced during the fermentation process due to the formation of a new theaflavin-type compound, theagallinin, which is the condensation product between EC and theagallin, the amount of gallic acid is significantly increased in black tea due to the desertification of the 3-galloyl-substituted catechins by either native esterase or oxidative degallation during fermentation. Gallic acid bioactive compound is famous for its biological and pharmacological activity so they contain antimicrobial activity, anticarcinogenic activity, anti-inflammatory activity, as well as showing gastroprotective neuroprotective and cardioprotective activity [96].

2.9.10 Propionic acid

In recent years, there has been increased interest in the bio production of propionic acid by Propionibacterium. Among all organic acids, propionic acid (PA) and its derivatives have received the most attention. It can be mentioned as important chemical intermediates that are generally utilized in a variety of industrial applications as antibacterial treatments for a wide range of microorganisms, anti-inflammatory substances with analgesic and antipyretic characteristics and so on [97].

2.10 Computer Aided Drug Designing

Computer aided drug designing is the inventive process of finding new drugs base on the knowledge of biological target the drug is most commonly organic small molecule that activate or inhibit the function of macro molecule such as proteins molecular docking is a structure based drug design method which predict the binding affinity and mode between receptor and ligand and stimulate the molecular interaction. So now a days this technology is extensively used in drug designing process [98].

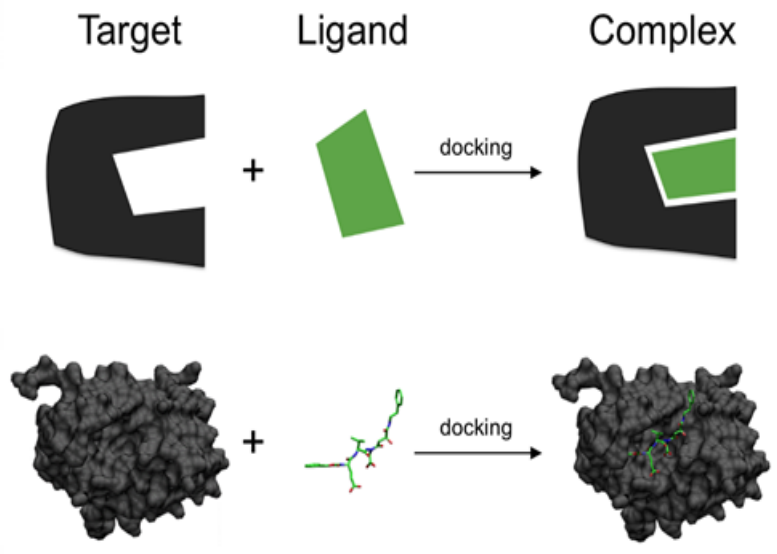


FIGURE 2.7: Diagram shows us complete molecular docking process [99].

There are different software's which are most efficiently used for molecular docking process such as CB dock, Auto dock, vina dock, Swiss dock and assisted molecular docking (AM dock).

In computational biology computer aided drug designing is an important step. So for our research study we follow all those computational tools which are necessary to design a drug. Molecular docking is a process which can estimate strength of binding between ligand and receptors.

Chapter 3

Materials and Methods

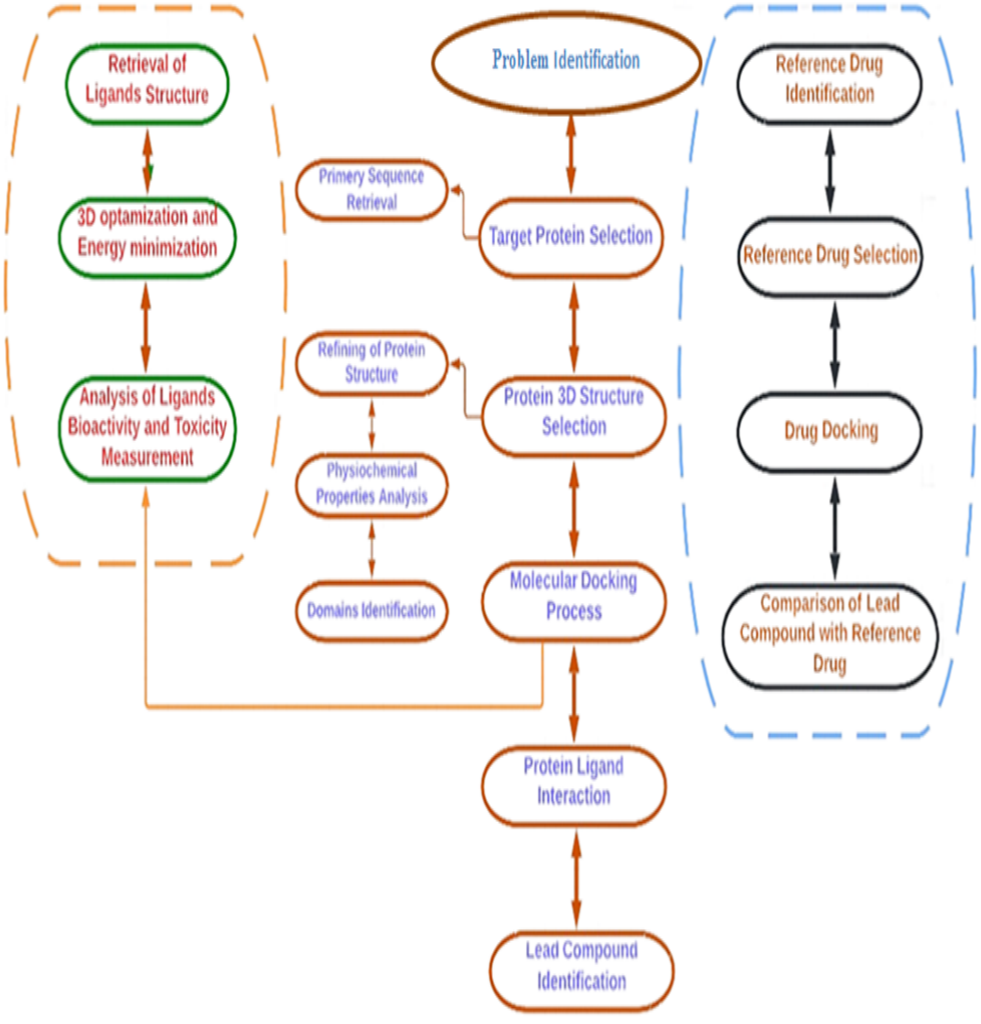


FIGURE 3.1: Flow chart of Methodology.

3.1 Problem Identification

Listeriosis is an uncommon but potentially fatal infection; caused by Listeria monocytogenes. This organism can be discovered. It can be found in soil, plants, and animals.

The main route of transmission is thought to be through the consumption of contaminated food. Infection can, however, occur and be transmitted, albeit rarely, directly from infected animals to humans as well as between humans [100]. L. monocytogenes can be transmitted in neonatal infections. In utero or during passage from mother to child birth canal was harmed.

3.2 Selection of Targeted Proteins

Through interaction with the human cellular receptor E-cadherin (hEcad), Internalin A promotes infection of human enterocyte-like cell lines [101]. The amino acid at position 16 of E-cadherin is species-specific and is critical for the InlA-E-cadherin interaction.

Internalin B has a wide range of host cell receptors and promotes invasion by activating phosphatidylinositol 3-kinase [102]. Met receptor tyrosines kinases have been identified as InlB-recognized host cell receptors.

Listeriolysin O is a hemolysin produced by the bacterium Listeria monocytogenes. LLO belongs to the cholesterol-dependent cytolysin family (CDCf) [103].

3.2.1 Retrieval of Primary Sequence

Target protein (Internalin A, Internalin B and Listeriolysin O) primary sequence were retrieve in Fasta format from UniProt database with accession number P0DJM-0, P0DQD3 and P13128, with residues length of 800AA,630AA and 529AA.

3.3 Retrieval of 3D Structure of Targeted Proteins

Protein databank is the only source of information regarding three dimensional structures of biological molecules such as proteins.

3D structures of targeted protein, Internalin A, Internalin B and Listeriolysin O with PDB ID 1O6S, 1H6T and 4CDB were obtained from protein databank.

3.3.1 Refining of Protein Structures

For refining process there is a lot of tools such as (discovery studio, Pymol and chimera x) which can be mostly used for cleaning of protein structures form water molecules and other complex molecules.

Pymol is open access software which is mostly use in computational research work to refine protein structure from water molecule and other complex molecules like ligands.

3.3.2 Physiochemical Properties Analysis

Physiochemical properties play a vital role in determining the chemical and physical function of proteins. ProtParam is an online server which was used to predict physiochemical properties of proteins such as Internalin A, Internalin B and Listeriolysin O.

The number of positively charged residue (Arg+Lys) and negatively charged residue (Asp+Glu), theoretical pI, molecular weight, instability index, aliphatic index.

Extinction coefficient 1 and extinction coefficient 2, grand average of hydrophobicity, so these parameters were computed through ProtParam online database [104].

3.3.3 Domains Identifications

Interpro is an online database which was most probably used to identify functional domain of targeted proteins (Internalin A, Internalin B and Listeriolysin O). By putting FASTA sequence of the protein in the Interpro database polypeptide binding site and homodimer interfaces were studies [105].

3.4 Retrieval of Ligands Structure

PubChem is globally largest database of easily accessible chemical information database. It contains information of biological molecules like proteins, lipids and carbohydrates and also modified macromolecules store in the form of chemical names, molecular formula, molecular weight, 2D and 3D structure of ligands, their isomers and canonical similes. The selected ligands 3D structures were retrieving from PubChem database in sdf format [106].

3.4.1 3D Optimization and Energy Minimization of Ligand

Three dimensional optimization and energy minimization of ligand were carried out by Chem pro ultra-software (v 12.0.2). Energy minimization is an important step the preparation of ligand molecule for docking process because unstable ligands will show inaccurate vina score in docking results.

3.5 Analysis of Ligands Bioactivity and Toxicity Measurement

Chemical compounds used as a ligand were virtually analyzed on the basis of Lipinski rule of five and those are used as an active drug in humans. The effectiveness of a compound is measured by ADMET properties. Swiss ADME and

PKCSM both are online tools which are used to predict ADMET properties of the compound [107]. The rules are listed below;

1. Hydrogen bond donor should be less than 5.
2. Hydrogen bond acceptor should be less than 10.
3. Molecular weight should be less than 500.
4. Log p value of molecule should be limited to 5.

3.6 Molecular Docking Process

Molecular docking is a process which is used to predict the most favorable conformational interaction between targeted protein and the selected compounds. For molecular docking process CB dock is an online blind docking tool used, which automatically predict the binding region of protein and by using coverture based cavity, detection method calculate size and center. The 1st step of docking process is to create a ligand and protein files. Pdb file of targeted protein (Internalin A, Internalin B and Listeriolysin O) were uploaded to CB dock as an input file. After completion of protein file then the ligand file has been prepared by following same procedure and uploaded in sdf format [108]. The results provided by CB dock was in five different pose of interaction among which the best pose was selected on the bases of minimum vina score and maximum cavity size and also maximum number of grid size value.

3.7 Protein Ligand Interaction

The interaction of the ligand and protein are calculated for the interpretation of docking results. Docked complex obtained in pdb format with lowest vina score was analyzed using the software Ligplot plus. Two type of interaction are studied, one is hydrogen bond interaction and the other is hydrophobic interaction. This

software automatically generate schematic 2D diagram of protein ligand interaction in the pdb file [109].

3.8 Lead Compound Identification

After a detail analysis of protein and ligand interaction, Docking score and ADMET properties studies, the most active inhibitor which fulfills Lipinski rule of five was identified. This selected compound was our lead compound.

3.9 Reference Drug Identification

Ampicillin, extended-spectrum penicillin, is effective against both Gram positive and Gram negative bacteria [110]. Gentamicin is an aminoglycoside antibiotic that is bactericidal. This works by inhibiting protein synthesis in sensitive microorganisms [111].

The reference drug ampicillin and gentamicin which is most probably use to treat listeriosis disease. Drug bank database are used for identification of reference drug like ampicillin and gentamicin, so these drugs are identified on the basis of physiochemical properties, ADMET properties, drug mechanism of action and less side effects.

3.10 Reference Drug Selection

The identified .drugs are filtered to select most effective drug. Drug bank database were used for drug selection because it helps to analyze the disease in detail with its pathways and drugs.

The ADMET properties and mechanism of action with drug side effects were obtained from pkCSM, drug bank and PubChem databases respectively.

3.11 Reference Drug Docking

The selected references drugs are docked with Internalin A, Internalin B and Listeriolysin O proteins to identify the cure of listeriosis. Docking process is performing through CB dock, which is an online docking server.

3.12 Comparison of Lead Compound with Reference Drug

The comparison between lead compound and reference drug is done by comparing docking results, physicochemical properties, ADMET properties and interaction properties.

Chapter 4

Results and Discussions

4.1 Structure Modeling

Our target proteins for Insilco study against Internalin A, Internalin B and Listeriolysin O were selected as inhibitory proteins against metabolic compounds which was identified from bifidobacterium *aquakifiri*.

These compounds include Bacteriocins, Acetyl phosphate, Fructose-6-phosphate, Cinnamyl alcohol, β-Caryophyllene Alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid, Propionic acid and Short chain fatty acid.

4.1.1 Primary Sequence Retrieval

Primary sequence retrieval of target proteins are an important step in drug designing process. So from these sequences we can identify physicochemical properties as well as functional domains of target proteins.

These protein sequence were obtain from UniProt database In FASTA format with accession number PODJMO, PODQD3, P13128 and residue length 800AA, 630AA, 529AA.

4.1.2 Proteins 3D Structure selection

3D structures of target proteins were obtained from protein databank in Pdb format. Protein databank is database of a complex molecule of living organism such as protein and nucleic acid. The target proteins for Insilco studies were Internalin A Internalin B and Listeriolysin O were downloaded from protein databank.

Pymol is protein visualization and refining software, with the help of this software which can easily refine protein structure from water molecules and our complex compounds. Figure 4.1, 4.2 and 4.3 shows us refine structure of Internalin A, Internalin B and Listeriolysin O respectively.

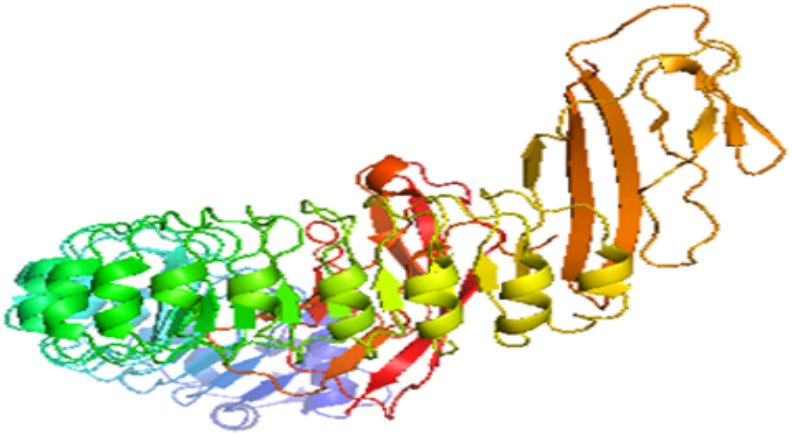


FIGURE 4.1: Refine 3D structure of Internalin A.

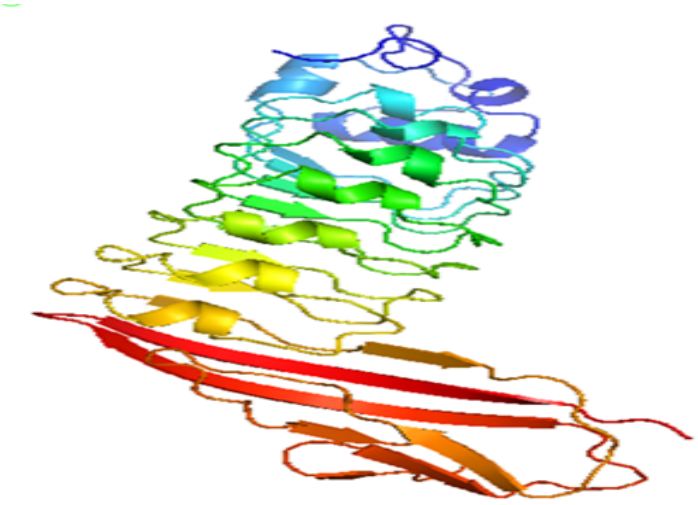


FIGURE 4.2: Refine 3D structure of Internalin B.

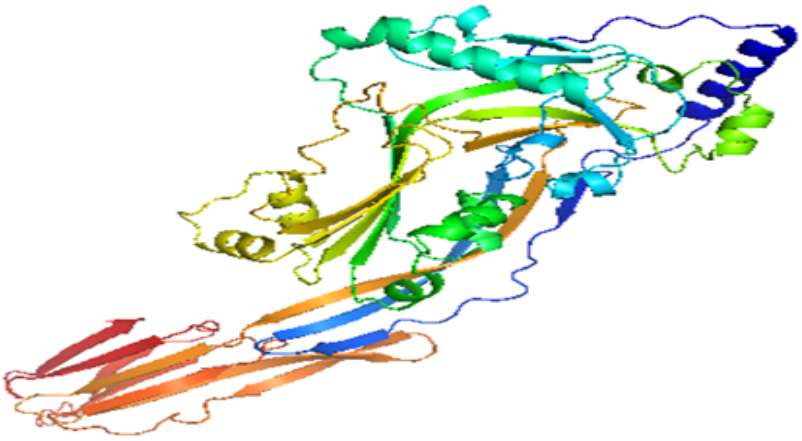


FIGURE 4.3: Refine 3D structure of Listeriolysin O.

4.1.3 Functional Domain Identification

Interpro database was used to identify classification of proteins family and to identify functional domain active site of Internalin A, Internalin B and Listeriolysin O. So functional domains are major parts of proteins having active sites utilized by protein to interact with other complex substances [112]. Protein has more than one functional domain which performs different functions. Internalin A protein contain three functional domain that are Internalin-N domain which starting from 7 amino acid and ending at 28 amino acid, LRR-contain-adjecent-domain which starting from 409 amino acid and ends with 465 amino acid and Cadherin-like-domain which start from 477 and end with 570 amino acids respectively. These domains are represented in different colors; first domain Internalin-N was shown in red color, LRR-contain-adjucent domain was shown in blue color while Cadherin-like-domain was shown in orange color in figure 4.4. Internalin B contain two functional domain with naming of Internalin-N domain starting from 6 amino acids and end with 28 amino acids and LRR-contain-adjucent domain which start from 234 amino acids and end with 290 amino acids, so these domains(Internalin-N and LRR-contain-adjucent-domain) are shown in different colors like yellow and magenta were represented in figure 4.5. Listeriolysin O contain only one functional domain which is Thiol-cytolys-c domain which start from 380 amino acids and end with 481 amino acids [113]. This domain thiol-cytolys-c shown in spectrum (rainbow) color which was shown in figure 4.6.

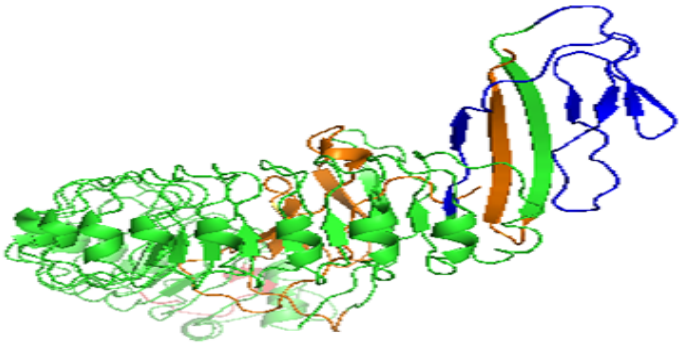


FIGURE 4.4: Internalin A protein contain three domains which are shown in blue, orange and red colors.

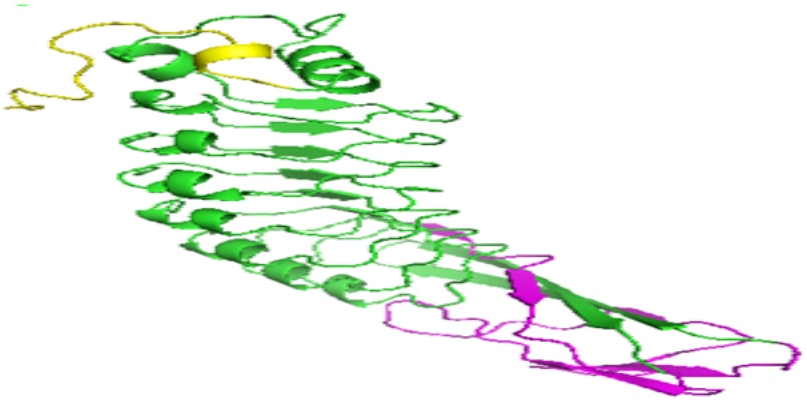


FIGURE 4.5: Internalin B protein contain two domains in yellow and magenta colors.

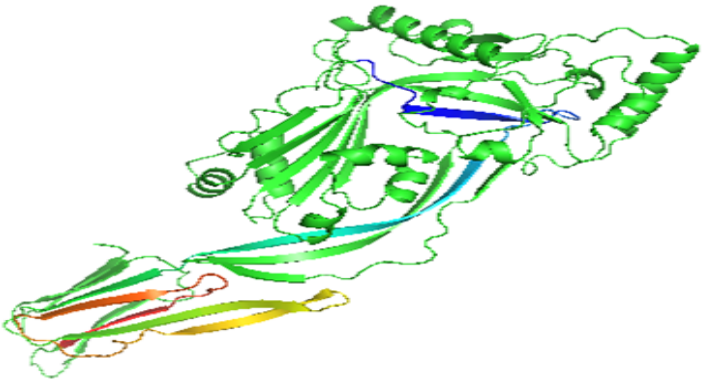


FIGURE 4.6: Listeriolysin O protein contain one functional domain which are shown in rainbow color.

4.1.4 Physiochemical Properties of Target Proteins

ProtParam is an online tool which is used to compute various physical and chemical properties for a given proteins stored in Swiss Prot to putting a protein sequence.

The compute parameters include molecular weight, theoretical pI, amino acid composition, extinction coefficient 1and 2, aliphatic index, instability index and grand average of hydrophobicity.

The theoretical pI shows us acidic and basic properties of proteins. While extinction coefficient shows us light absorption, whereas instability index represent us protein stability in nature.

So if the value of proteins stability is less than 40 it means that protein is stable while this value is greater than 40 then it shows us protein instability [114].

TABLE 4.1: Physicochemical properties of Internalin A.

S No	Parameters	Internalin A
1	Number of amino acids	800
2	Molecular weight	86492.91
3	Theoretical pI	4.93
4	Negatively charged residue (Asp+Glu)	72
5	Positively charged residue (Arg+Lys)	58
6	Ext.Co 1	96260
7	Instability Index	20.52
8	Aliphatic index	88.31
9	GRAVY	-0.298

Table 4.2: Physicochemical properties of Internalin B.

S No	Parameters	Internalin B
1	Number of amino acids	630
2	Molecular weight	71220.64
3	Theoretical pI	9.58
4	Negatively charged residue (Asp+Glu)	61
5	Positively charged residue (Arg+Lys)	91
6	Ext.Co 1	107970
7	Ext.Co 2	107720
8	Instability Index	21.29
9	Aliphatic index	89.75
10	GRAVY	-0.466

Table 4.3: Physicochemical properties of Listeriolysin O.

S No	Parameters	Listeriolysin O
1	Number of amino acids	529
2	Molecular weight	58688.10
3	Theoretical pI	7.63
4	Negatively charged residue (Asp+Glu)	59
5	Positively charged residue (Arg+Lys)	60
6	Ext.Co 1	75750
7	Instability Index	34.71
8	Aliphatic index	84.59

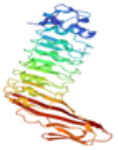
9	GRAVY	-0.470

The alipathic index represent the aliphatic content of the protein. So the greater value of aliphatic index indicates the thermo stability of the protein. Molecular weight of the target protein contains both positive and negatively charge residues. Low GRAVY shows better interaction with water molecules.

4.2 Template Selection

Protein databank is large repository database which is mostly used to retrieve biological and macromolecules like proteins and nucleic acids. The simplest template selection rule is to choose the structure that matches to the model sequence. 3D structure of were taken from protein databank which were listed in table 4.4.

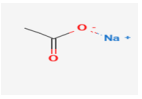
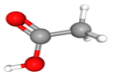
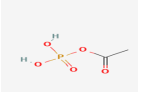
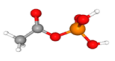
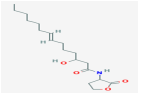
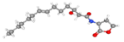
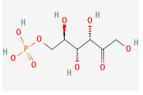
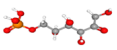
TABLE 4.4: Following table shows us proteins pdb id, resolution and 3D structure.

S.No	**Templates**	**Resolution**	**PDB ID**	**3D-Structures**
1	Internalin"(Listeria monocytogenes) / E-Cadherin (human) Recognition Complex".	1.8 Å	1O6S	
2	Internalin B:"crystal structure of fused N-terminal domains".	1.60 Å	1H6T	
3	Crystal structure of Listeriolysin O.	2.15 Å	4CDB	

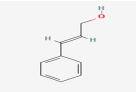
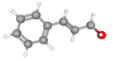
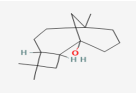
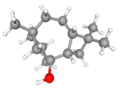
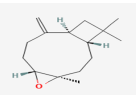
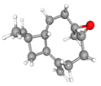
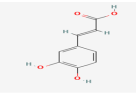
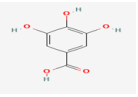
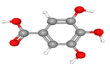
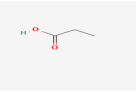
4.3 Ligand Selection

The selection of ligands which based on the best resolution of the structure, the chemical class of the co-crystal ligands bound to the protein structure with best binding affinity. Ligands were selected from PubChem database, which is globally freely accessible chemical information database. The ligand 3D structure downloaded from PubChem database in sdf format [115]. After selection of ligands we used energy minimization via Chem pro 3D software which is easily available. Energy minimization and 3D optimization is an important step in the preparation of ligand compounds for docking process because unstable ligands will show inaccurate vina scores. Metabolic compounds were selected as ligands for the present Insilco work [116]. The 3D structure and chemical information were retrieve from PubChem data base and the selected ligands are short chain fatty acids, acetyl phosphate, Bacteriocin, fructose-6-phosphate, Cinnamyl alcohol, β-Caryophyllene alcohol, Caryophyllene oxide, Caffeic acid, gallic acid, propionic acid.

TABLE 4.5: Given table represent us ligands 2d, 3D structures, molecular formula and molecular weight.

S no	Compounds	Molecular Formula	Molecular weight	2D-Structure	3D-Structure
1	Short chain fatty acids	$C_2H_3NaO_2$	82.03 g/-mol		
2	Acetyl phosphate	$C_2H_5O_5P$	140.03g/mol		
3	Bacteriocins	$C_{18}H_{31}NO_4$	325.4g/mol		
4	Fructose-6-phosphate	$C_6H_{13}O_9P$	260.14g/mol		

Continue Table 4.5: Given table represent us ligands 2d, 3D structures, molecular formula and molecular weight.

S no	Compounds	Molecular Formula	Molecular weight	2D-Structure	3D-Structure
5	Cinnamyl alcohol	$C_9H_{10}O$	222.37g/mol		
6	β/Caryophyllene/alcohol	$C_{15}H_{26}O$	222.37g/mol		
7	Caryophyllene oxide	$C_{15}H_{24}O$	220.35g/mol		
8	Caffeic acid	$C_9H_8O_4$	180.16g/mol		
9	Gallic acid	$C_7H_6O_5$	170.12g/mol		
10	Propionic acid	$C_3H_6O_2$	74.08g/mol		

4.4 Virtual Screening of a Selected Ligands

Drug like and non-drug like compounds are separated by following certain parameter like Lipinski rule of five and ADMET properties. So the rule of five contain four parameters such as molecular weight, hydrogen bond acceptor, hydrogen bond donor and log p value these parameters are associated with active compounds. The meaning of drug likeness is depending upon mode of administration. A compound considered has a drug likeness when it complying with following three or more rule of five's. If a compound does not follow these rules so it can consider being poorly has absorbed drug [117]. There are a lot of tools which can be mostly used to check ADMET properties of a drug. pkCSM is an online tool which is used to

calculate the ADMET properties of a compound or a drug. Toxicity of a different metabolic compound can be calculated by using pkCSM tool, so for this different methods are used to screen ligands and to check if a ligand is toxic or non-toxic. We identify ten metabolic compound for our Insilco study like include Bacteriocins, Acetyl phosphate, Fructose-6-phosphate,Cinnamylalcohol, β-Caryophyllene Alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid, Propionic acid and Short chain fatty acid and all of these metabolic compounds follow Lipinski rule of five's. so the normal rang value of Lipinski rule for a drug molecular weight are 500 or less than five hundred, log p value five or less than five, hydrogen bond acceptor value should be ten or less than ten and hydrogen bond donor for a normal drug should be five or less than five [118]. Applicability of Lipinski rule on selected ligands were listed in the below table 4.6.

Table 4.6: Applicability of Lipinski Rule on Selected Ligands

S.No	**Ligands**	**logP Value**	**Molecular Weight**	**H-Bond Acceptor**	**H-bond Donor**
1	Short chain fatty acids	-4.2398	82.034 g/mol	2	0
2	Acetyl phosphate	-0.3578	140.031 g/mol	3	2
3	Bacteriocins	3.5097	463.464 g/mol	7	3
4	Fructose-6-phosphate	-3.2602	260.135 g/mol	7	6
5	Cinnamyl alcohol	-1.6921	134.178 g/mol	1	1
6	β/Caryophyellene alcohol	-3.7539	222.372 g/mol	1	1
7	Caryophyllene oxide	-3.9364	220.356 g/mol	1	0
8	Caffeic acid	-1.1956	180.159 g/mol	3	3
9	Gallic acid	-0.5016	170.12 g/mol	4	4
10	Propionic acid	-0.481	74.079 g/mol	1	1

4.5 Toxicity Predication of Selected Compounds

In drug designing toxicity predication is very important property to check and evaluate the toxicity of a drug before drug designing. So for admet properties and toxicity prediction pkCSM is an online tool which is most frequently used to predict and analyze admet properties and toxicity prediction.

AMES toxicity are those toxicity which having carcinogenic effect on the body, so if we compare over all toxicity results of selected metabolic compounds so these compounds such as short chain fatty acids, acetyl phosphate, Bacteriocins, fructose-6-phosphate, caffic acid, gallic acid and propionic acid having no AMES toxicity and hepatotoxicity but if we look at skin sensitization property.

AMES Toxicity (Salmonella typhimurium reverse mutation assay) uses bacteria to find the mutagenic potential of the compound. Positive response indicates that ligand is mutagenic in the DNA of test organism and can also act as a carcinogen.

So the three compounds such as Cinnamyl alcohol, beta-Caryophyllene and Caryophyllene oxide showing skin sensitization [119]. Hepatotoxicity are those toxicity which damage liver properly by taking drugs which having high hepatotoxicity level so hepatotoxicity is a major safety concern for drug development.

Skin sensitivity is a potential side effect of having drugs which shows sensitivity to skin. The hERG I and II inhibitors is said to cause the inhibition of potassium channels induced by hERG, so these are the main cause of chronic syndrome leading to fatal ventricular arrhythmia.

The toxicity values mentioned in the table shows that on the basis of toxicity tests like skin sensitization, hERG II inhibitor, Minnow toxicity we can screen out all selected compounds which pass the toxicity test, but final screening whould be based on ADME properties. The toxicity predictions of the metabolic compounds were given in below table 4.7.

Table 4.7: Toxicity predication of selected ligands.

Ligands	**AMES toxicity**	**Max. tolerated dose (human)**	**hERG I Inhibitor**	**hERG II Inhibitor**	**Oral rat acute toxicity**	**Oral rat chronic toxicity**	**Hepato-tocicity**	**Skin sensi-tization**	**T. pyri-formis-toxicity**	**Min-now toxicity**
Short-Chain-Fatty-Acid	No	1.073	No	No	1.337	-1.559	No	No	-0.123	3.049
Acetyl-Phos-phate	No	1.356	No	No	1.879	2.617	No	No	0.232	2.878
Bacter-iocins	No	0.329	No	No	2.245	2.662	No	No	0.285	1.913
Fructose-6-Phos-phate	No	1.631	No	No	1.65	3.886	No	No	0.285	6.308
Cinnamyl-alcohol	No	0.936	No	No	1.92	1.835	No	Yes	-0.125	1.873

Continue Table 4.7: Toxicity predication of selected ligands.

Ligands	**AMES toxicity**	**Max. tolerated dose (human)**	**hERG I Inhibitor**	**hERG II Inhibitor**	**Oral rat acute toxicity**	**Oral rat chronic toxicity**	**Hepato-tocicity**	**Skin sensi-tization**	**T. pyri-formis-toxicity**	**Min-now toxicity**
β-Caryo-phyellne-alcohol	No	0.206	No	No	1.756	1.215	No	Yes	1.005	1.094
Caryop-hyllene-Oxide	No	0.148	No	No	1.548	1.224	No	Yes	1.079	0.955
Caffeic-acid	No	1.145	No	No	2.383	2.092	No	No	0.293	2.246
Gallic-acid	No	0.7	No	No	2.218	3.06	No	No	0.285	3.188
Propionic-acid	No	1.046	No	No	1.776	2.603	No	No	-0.865	2.44

4.6 Molecular Docking

In computational biology molecular docking is a process which is used to determine the strength of binding between ligand and proteins at atomic level. Molecular docking is a process of structure base drug designing which predicts the binding affinity between receptor and ligand complexes and simulates the molecular interaction. So this technology is extensively used in computer aided drug designing research. The 3D structure of ligands and protein structure were taken has input file for molecular docking. CB dock is an online docking software which is used for docking purpose. CB dock predict the binding sites of proteins and calculate the cavity size through auto dock vina [120]. The results and time required for docking process is totally depend on receptor structure, energy minimization of ligands and speed of net connectivity. CB dock gives us out put size of five"best poses and models for receptors, the best poses select on the basis of vina score and cavity size. Molecular docking process were performed by using receptors such as Internalin A, Internalin B, Listeriolysin O and ten selected compounds as a ligands. So these are submitted as a input file in pdb and sdf format. Among five this confirmation less vina scores pose were selected for protein ligand interaction. Ligands with best docking score were shown in table 4.8.

Table 4.8: Following table shows us ligands with docking results with Internalin A.

S.No	**Ligands**	**Binding Score**	**Cavity size**	**Grid Map**	**HBA and HBD**	
1	Short chain Fatty acid	- 3.6	2017	26	2	0
2	Acetyl Phosphate	-4.3	2017	35	3	2
3	Bacteriocins	-6.4	2017	59	7	3
4	Fructose-6-Phosphate	-6	2017	59	7	6
5	Cinnamyl alcohol	-5.9	2017	34	1	1

Continue Table 4.8: Following table shows us ligands with docking results with Internalin A.

S.No	**Ligands**	**Binding Score**	**Cavity size**	**Grid Map**	**HBA and HBD**	
6	β/Caryopylene alcohol	- 5.5	2017	34	1	1
7	Caryophyllene oxide	-5.8	2017	33	1	0
8	Caffic acid	-6.4	2017	59	3	3
9	Gallic acid	-6.2	2017	59	4	4
10	Propionic acid	-3.7	2017	26	1	1

Continue TABLE 4.8: Following table shows us ligands with docking results with Internalin A.

S.No	**Ligands**	**M.W (g/mol)**	**LogP**	**Max-Energy (kcal/-mol)**	**Min Energy (kcal/-mol)**
1	Short chain Fatty acid	82.034	-4.2398	2.3964	0.4098
2	Acetyl-Phosphate	140.031	-0.3578	19.2493	-8.4906
3	Bacteriocins	463.464	3.5097	5.9978	-6.6494
4	Fructose-6-Phosphate	260.135	-3.2602	19.4924	-12.2896
5	Cinnamyl alcohol	134.178	1.6921	0.8299	0.0297
6	β-Caryophyllene alcohol	222.372	3.7539	59.5184	0.0000
7	Caryophyllene oxide	220.356	3.9364	87.9156	-1.2779
8	Caffic acid	180.159	1.1956	5.0475	0.3697
9	Gallic acid	170.12	0.5016	11.5108	-41.1855
10	Propionic acid	74.079	0.481	1.1518	0.3913

Internalin B is the protein which is found in listeria monocytogenes and this protein is most probably responsible to cause listeriosis disease in neonatal baby.so Internalin B protein over expression can also responsible for listeriosis disease.to control the over expression of this protein we can design metabolic compound

through computer aided drug designing process which can significantly inhibit the over expression of this protein. The basic theory of molecular docking is to simulate the optimal confirmation according to complementary and pre organization which could obtain and predict the binding affinity and interaction mode of ligand and receptors.

The following table 4.9 shows us Internalin B docking score, cavity size, grid map values, hydrogen bond acceptor, hydrogen bond donor, log p value, minimum energy and maximum energy values.

TABLE 4.9: Following table shows us ligands with docking results with Internalin B.

S.No	**Ligands**	**Binding Score**	**Cavity size**	**Grid Map**	**HBA and HBD**	
1	Short chain Fatty acid	- 3	560	26	2	0
2	Acetyl Phosphate	-3.3	352	16	3	3
3	Bacteriocins	-5.1	352	49	7	3
4	Fructose-6-Phosphate	-5.4	560	26	7	6
5	Cinnamyl alcohol	-4.4	352	49	1	1
6	β/Caryopylene alcohol	- 5	560	26	1	1
7	Caryophyllene oxide	-5.4	560	26	1	0
8	Caffic acid	-5.2	560	26	3	3
9	Gallic acid	-4.8	560	26	4	4
10	Propionic acid	-3.3	560	26	1	1

Continue TABLE 4.9: Following table shows us ligands with docking results with Internalin B.

S.No	**Ligands**	**M.W (g/mol)**	**LogP**	**Max-Energy (kcal/-mol)**	**Min Energy (kcal/-mol)**
1	Short chain Fatty acid	82.034	-4.2398	2.3964	0.4098
2	Acetyl-Phosphate	140.031	-0.3578	19.2493	-8.4906
3	Bacteriocins	463.464	3.5097	5.9978	-6.6494
4	Fructose-6-Phosphate	260.135	-3.2602	19.4924	-12.2896
5	Cinnamyl alcohol	134.178	1.6921	0.8299	0.0297
6	β-Caryophyllene alcohol	222.372	3.7539	59.5184	0.0000
7	Caryophyllene oxide	220.356	3.9364	87.9156	-1.2779
8	Caffic acid	180.159	1.1956	5.0475	0.3697
9	Gallic acid	170.12	0.5016	11.5108	-41.1855
10	Propionic acid	74.079	0.481	1.1518	0.3913

Listeriolysin O is the protein which is also responsible to cause listeriosis. So in this research work we focus on the inhibition of over expression of this protein through metabolic compounds which is identified from Bifidobacterium *aquikifir*.

So these metabolic compound are used as a drug candidate to inhibit the activity of this protein through molecular docking process. Molecular docking process is an important steps during computer aided drug designing. The following table -

4.10 shows us target protein docking score, cavity size, grid map values, hydrogen bond acceptor, hydrogen bond donor, log p value, minimum energy and maximum energy values.

TABLE 4.10: Following table shows us ligands with docking results with Listeriolysin O.

S.No	**Ligands**	**Binding Score**	**Cavity size**	**Grid Map**	**HBA and HBD**	
1	Short chain Fatty acid	- 3.4	493	20	2	0
2	Acetyl Phosphate	-4.1	493	16	3	3
3	Bacteriocins	-6.2	983	27	7	3
4	Fructose-6-Phosphate	-5.6	983	26	7	6
5	Cinnamyl alcohol	-5.6	983	29	1	1
6	β/Caryopylene alcohol	- 5.9	860	27	1	1
7	Caryophyllene oxide	-6.2	860	27	1	0
8	Caffic acid	-5.9	983	29	3	3
9	Gallic acid	-5.4	983	29	4	4
10	Propionic acid	-3.6	983	23	1	1

The following continue table 4.10 shows us Listeriolysin O molecular weight (g/-mol) value, log p value, minimum energy (kcal/mol) and maximum energy (kcal/-mol) values.

Continue TABLE 4.10: Following table shows us ligands with docking results with Listeriolysin O.

S.No	**Ligands**	**M.W (g/mol)**	**LogP**	**Max-Energy (kcal/-mol)**	**Min Energy (kcal/-mol)**
1	Short chain Fatty acid	82.034	-4.2398	2.3964	0.4098
2	Acetyl-Phosphate	140.031	-0.3578	19.2493	-8.4906
3	Bacteriocins	463.464	3.5097	5.9978	-6.6494
4	Fructose-6-Phosphate	260.135	-3.2602	19.4924	-12.2896
5	Cinnamyl alcohol	134.178	1.6921	0.8299	0.0297
6	β-Caryophyllene alcohol	222.372	3.7539	59.5184	0.0000
7	Caryophyllene oxide	220.356	3.9364	87.9156	-1.2779
8	Caffic acid	180.159	1.1956	5.0475	0.3697
9	Gallic acid	170.12	0.5016	11.5108	-41.1855
10	Propionic acid	74.079	0.481	1.1518	0.3913

4.7 Interaction of Ligands and Targeted Proteins

The Docking analysis were performed by using Ligplot plus (version v.1.4.5) and by using Pymol (version 1.7.4.5). This software automatically generate 2D graphically interaction [121]. These 2D diagram shows us hydrogen bond interactions,

hydrophobic interactions and protein ligand interaction. The 2D diagram of best binding scores of ligands the respective protein.

4.7.1 Interaction of metabolic compounds with Internalin A

Internalin A protein 3D structure were taken from protein databank database for molecular docking process so after molecular docking process we can check interaction between Internalin A and different selected metabolites. The interaction between inernalin A and metabolic compounds were performed by using Ligplot plus software which can generate results in the form of 2D schematic representation. Internalin A were shown in figure 4.7 to 4.16 while hydrogen bond interaction and hydrophobic interaction with Internalin A was listed in table 4.11.from figure 4.7 to 4.16 shows interaction of ligands with short chain fatty acids, acetyl phosphate, Bacteriocin, fructose-6-phosphate, cinnamylalcohol, β-Caryophyllene alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid, propionic acid with Internalin A protein.

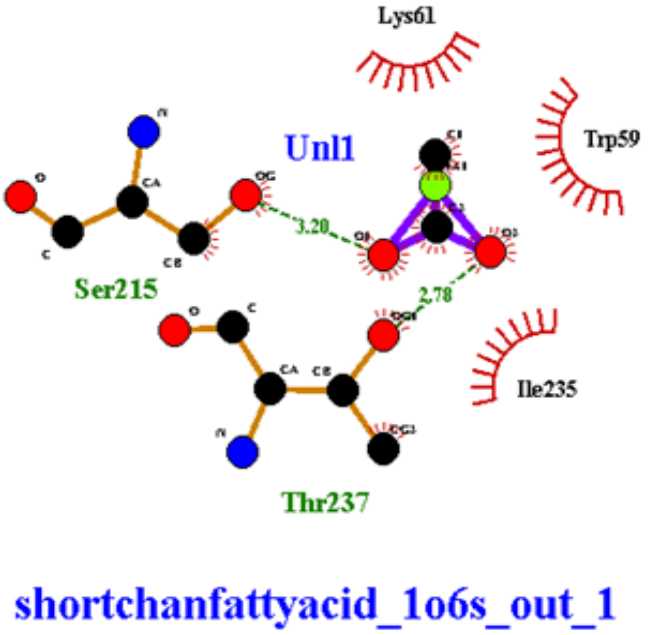


FIGURE 4.7: Interaction of short chain fatty acids with Internalin A

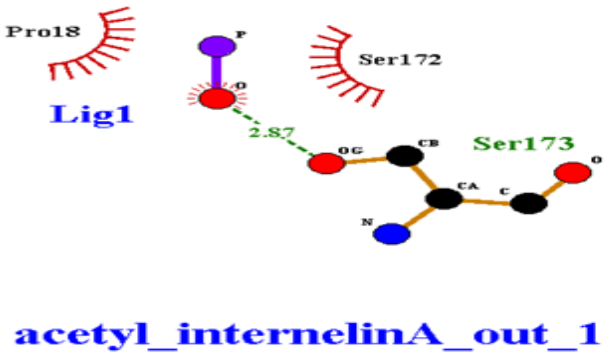


FIGURE 4.8: Interaction of Acetyl phosphate with Internalin A

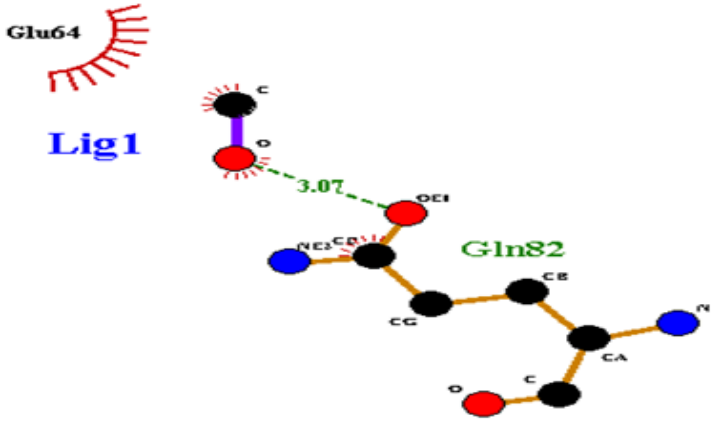


FIGURE 4.9: Interaction of Bacteriocin with Internalin A

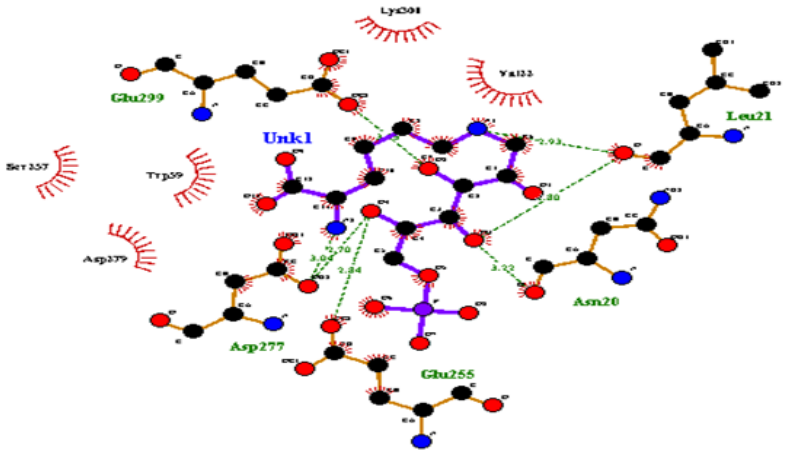


FIGURE 4.10: Interaction of Fructose 6 phosphate with Internalin A

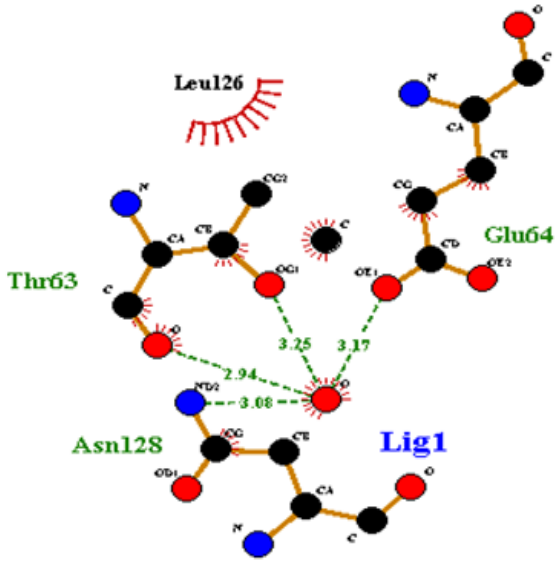


FIGURE 4.11: Interaction of Cinnamyl alcohol with Internalin A

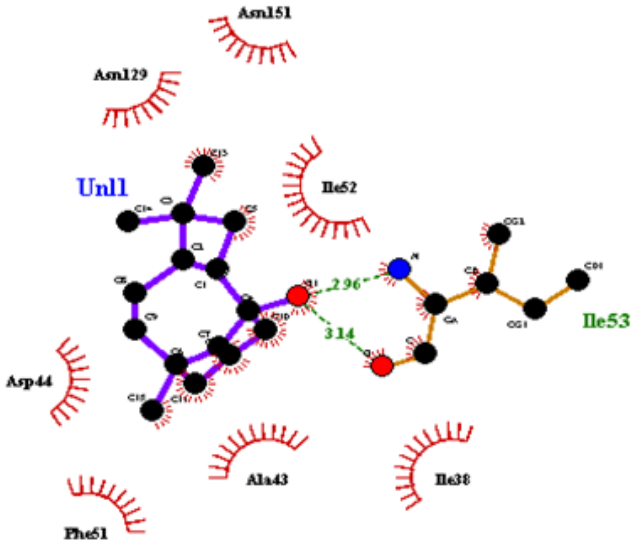
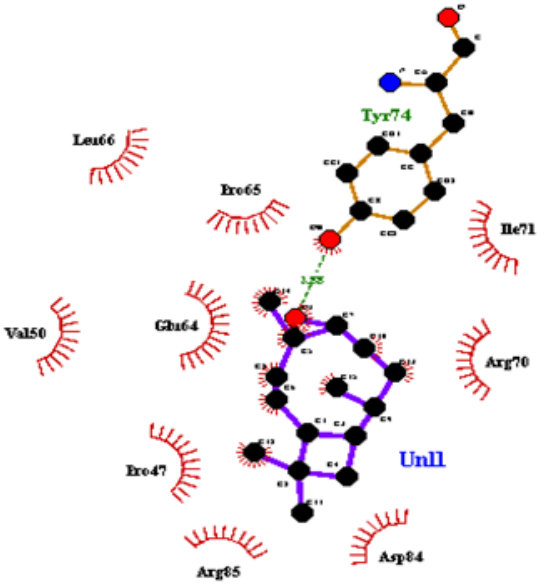
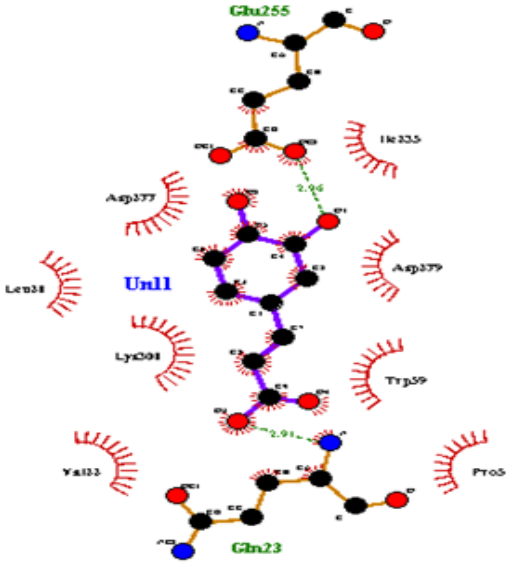


FIGURE 4.12: Interaction of β-Caryophyllene alcohol with Internalin A



Caryophylleneoxide_internelinA_out_1

FIGURE 4.13: Interaction of Caryophyllene oxide with Internalin A



caffeic_internelinA_out_1

FIGURE 4.14: Interaction of Caffeic acid with Internalin A

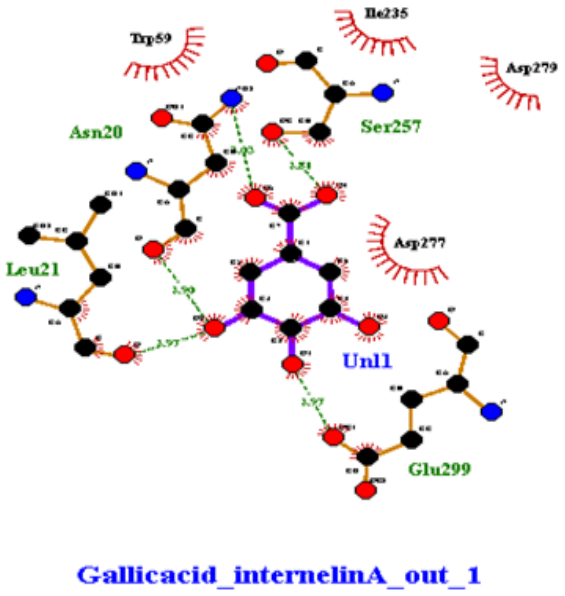


FIGURE 4.15: Interaction of Gallic acid with Internalin A

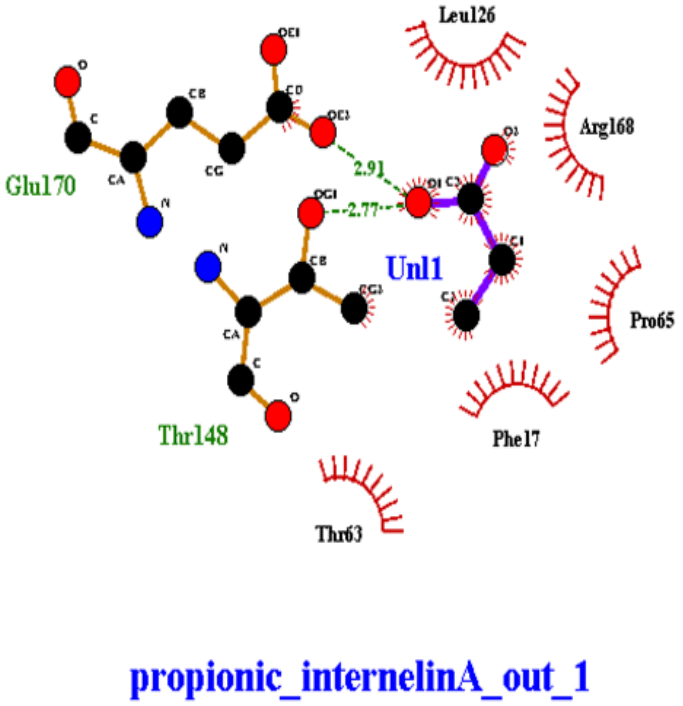


FIGURE 4.16: Interaction of Propionic acid with Internalin A

Table 4.11: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin A.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Short chain fatty acid	-3.6 & 2	Ser215 Thr237	3.20 2.78	Lys61 Trp59 Ile235
2	Acetyl phosphate	-4.3 & 1	Ser173	2.87	Pro18 Ser172
3	Bacteriocins	-6.4 & 1	Gln82	3.07	Glu64
4	Fructose-6-phosphate	-6.0 & 7	Glu299 Leu21 Asn20 Glu255 Asp277	3.3 2.70 3.04 2.84 3.22 2.30 2.93	Lys414 Val33 Ser357 Trp59 Asp379
5	Cinnamyl alcohol	-5.9 & 3	Thr63 Glu64	3.08 3.25 3.17	Leu126

Continue Table 4.11: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin A.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
6	β-Caryophyllene alcohol	-5.5 & 2	Ile53	2.96 3.14	Ile38 Ala43 Asp44 Phe51 Asn129 Asn151
7	Caryophyllene oxide	-5.8 & 1	Tyr74	2.33	Pro47 Val50 Glu64 Ale71 Asp84 Arg85
8	Caffeic acid	-6.4 & 2	Glu255 Gln23	2.96 2.91	Ile235 Asp279 Leu31 Asp277

Continue Table 4.11: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin A.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
9	Gallic acid	-6.2 & 5	Ser257 Glu299	3.03 2.81 2.90 2.93 2.93	Trp59 Ile235 Asp279 Asp277
10	Propionic acid	-3.7 & 2	Glu170 Thr148	2.91 2.77	Leu126 Arg168 Pro65 Phe17 Thr63

If we look at the overall hydrogen bonding interaction and hydrophobic interaction of all metabolic compounds so fructose-6-phosphate and Gallic acid are only two metabolic compounds which shows strong hydrogen bonding hydrophobic interaction as compared to other compounds.fructose-6-phosphate make 7 hydrogen bonding interaction and having five hydrophobic interaction with Internelin A. on the other hand Gallic acid make five hydrogen bonding interaction and four hydrophobic interaction with same protein Internelin and the rest of all metabolic compounds contain less hydrogen bonding and hydrophobic interaction.

4.7.2 Interaction of metabolic compounds with Internalin B

The interaction of metabolic compounds with best binding score with target protein Internalin B were shown in below table 4.12. Metabolic compound such as fructose-6-phosphate, gallic acid, Caffeic acid were shown strong interaction with Internalin B in these interaction they shows us hydrogen bond interaction and hydrophobic interaction.so these hydrophobic interaction means that the water molecules were removed during this interaction.

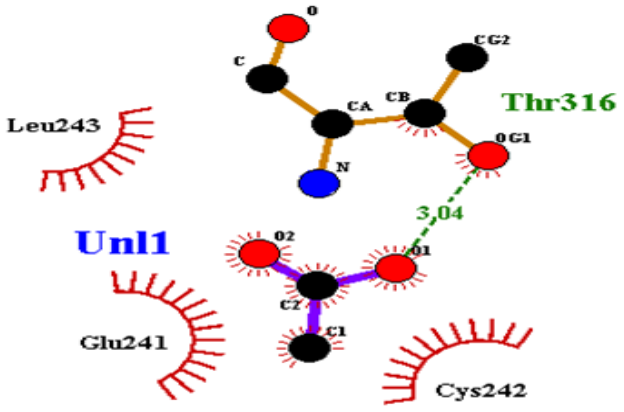


FIGURE 4.17: Interaction of short chain fatty acids with Internalin B

FIGURE 4.18: Interaction of Acetyl phosphate with Internalin B

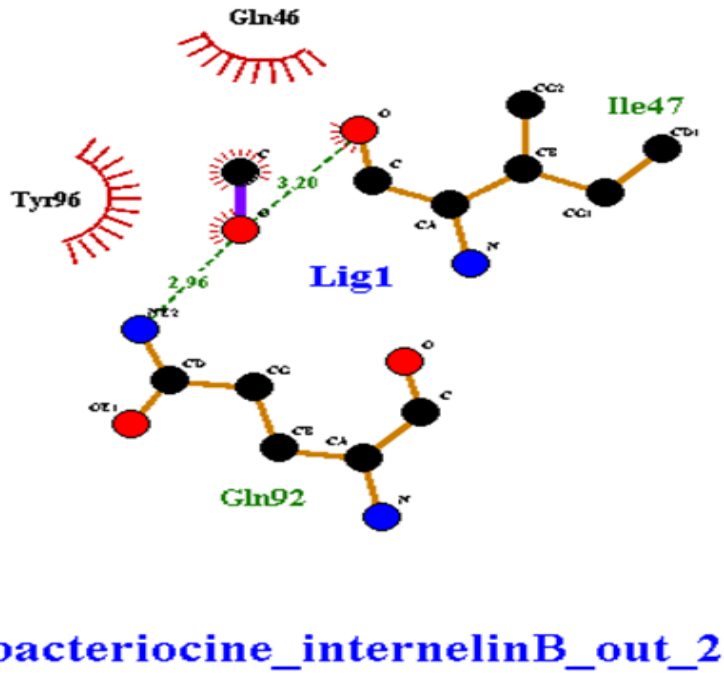


FIGURE 4.19: Interaction of Bacteriocin with Internalin B

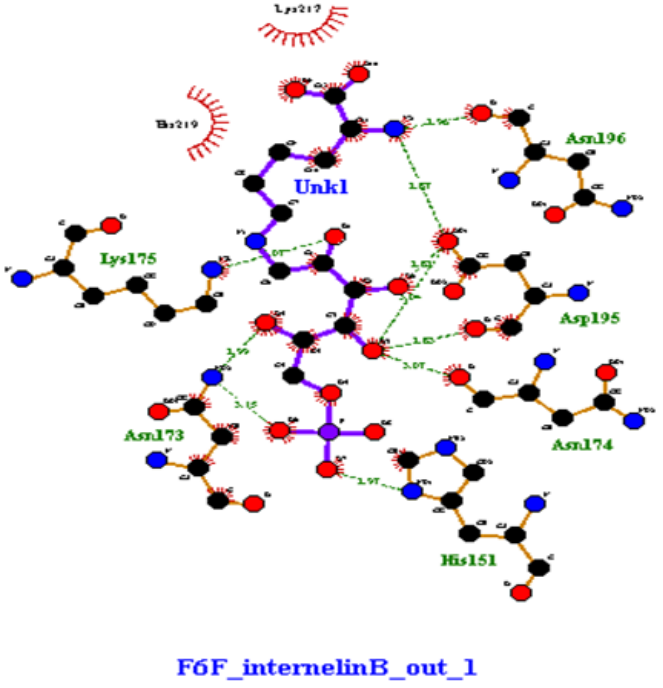


FIGURE 4.20: Interaction of Fructose 6 phosphate with Internalin B

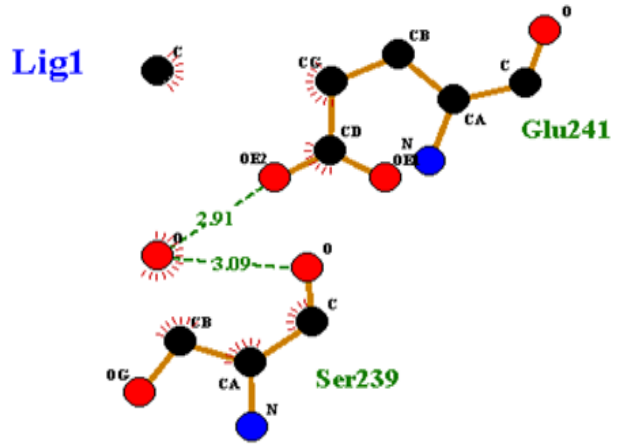


FIGURE 4.21: Interaction of Cinnamyl alcohol with Internalin B

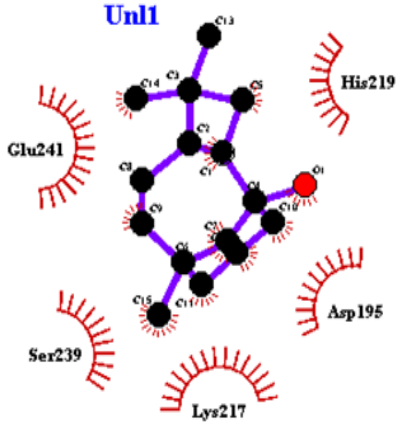


FIGURE 4.22: Interaction of β-Caryophyllene alcohol with Internalin B

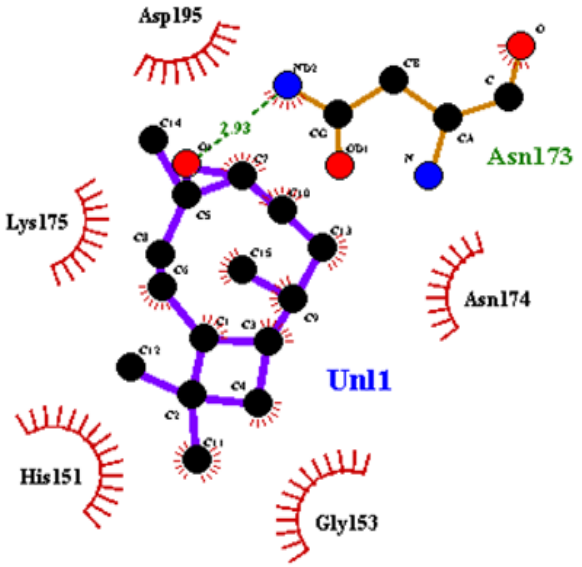


FIGURE 4.23: Interaction of Caryophyllene oxide with Internalin B

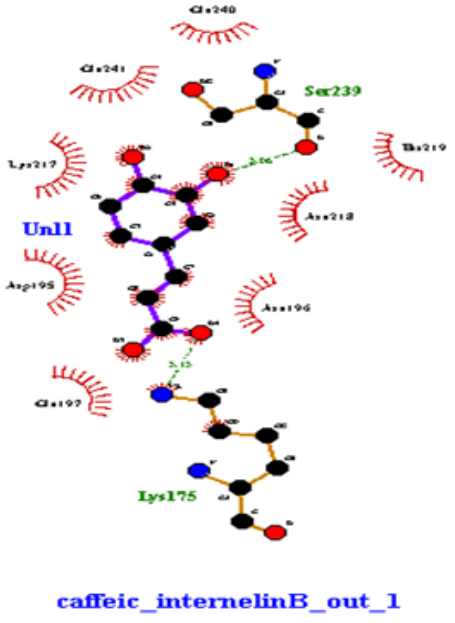


FIGURE 4.24: Interaction of Caffeic acid with Internalin B

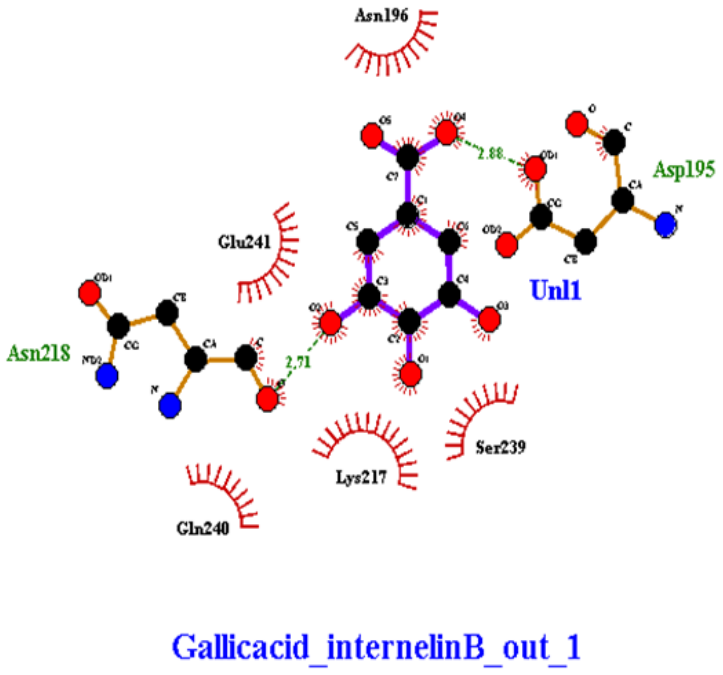


FIGURE 4.25: Interaction of Gallic acid with Internalin B

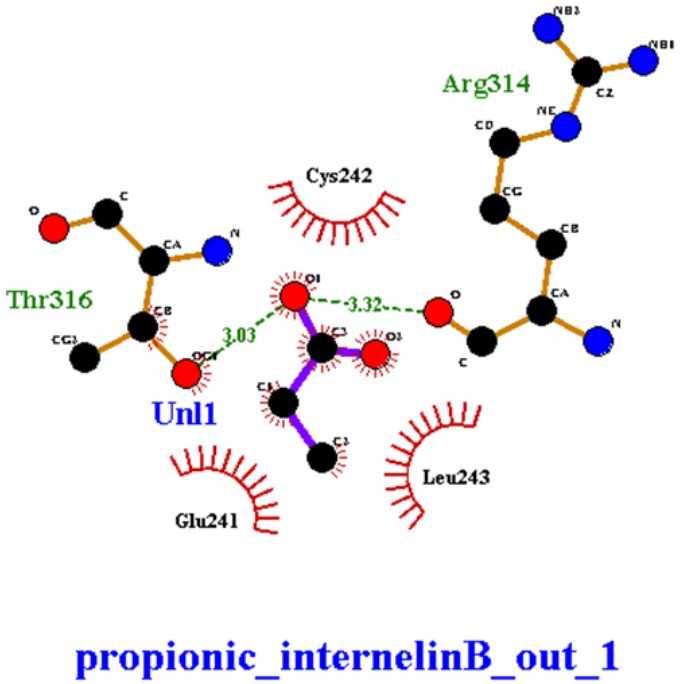


FIGURE 4.26: Interaction of Propionic acid with Internalin B

Table 4.12: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin B.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Short chain fatty acid	-3.0 & 1	Thr316	3.04	Glu41 Cys242 Leu243
2	Acetyl phosphate	-3.3 & —	—	—	His151
3	Bacteriocins	-5.1 & 2	Ile47	2.26	Gln46
4	Fructose-6-phosphate	-5.4 & 10	Gln92 His151 Asn173 Asn174 Lys175 Asp195 Asn196	3.20 2.83 2.87 2.93 2.99 3.03 3.04 3.07 3.15 3.83 3.90	Tyr96 Lys217 His219

Continue Table 4.12: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin B.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
5	Cinnamyl alcohol	-4.4 & 2	Ser239 Glu241	2.91 3.09	— —
6	β-Caryophyllene alcohol	-5.0 & —	UnI1	—	Asp195 Lys217 His219 Ser239 Glu241
7	Caryophyllene oxide	-4.9 & 1	Asn173	2.93	His151 Gly153 Asn174 Asp195
8	Caffeic acid	-5.2 & 2	Lys175 Ser239	3.06 3.13	Asp195 Asn196 Gln197 Lys217 His219

Continue Table 4.12: Active Ligand showing Hydrogen and Hydrophobic Interactions with Internalin B.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
					Gln240
					Gln241
9	Gallic acid	-4.8 & 2	Asp195	2.71	Asn196
			Ans218	2.88	Lys217
					Ser239
					Gln240
				Glu241	
10	Propionic acid	-3.3 & 2	Arg314	3.03	Glu241
			Thr316	3.32	Cys242
					Leu243

If we look at the overall hydrogen bonding interaction and hydrophobic interaction of all metabolic compounds so fructose-6-phosphate and Caffeic acid are only two metabolic compounds which shows strong hydrogen bonding hydrophobic interaction as compared to other compounds.fructose-6-phosphate make 10 hydrogen bonding interaction and having two hydrophobic interaction with Internalin B. on the other hand Caffeic acid make 2 hydrogen bonding interaction and eights hydrophobic interaction with same protein Internalin and the rest of all metabolic compounds contain less hydrogen bonding and hydrophobic interaction.

4.7.3 Interaction of metabolic compounds with Listeriolysin O

The interaction of metabolic compounds with best binding score with target protein Listeriolysin O were shown in below table 4.13. Metabolic compound such as fructose-6-phosphate, gallic acid, Caroleophyllene oxide were shown strong interaction with Listeriolysin O in these interaction they shows us hydrogen bond interaction and hydrophobic interaction.so these hydrophobic interaction means that the water molecules were removed during this interaction.

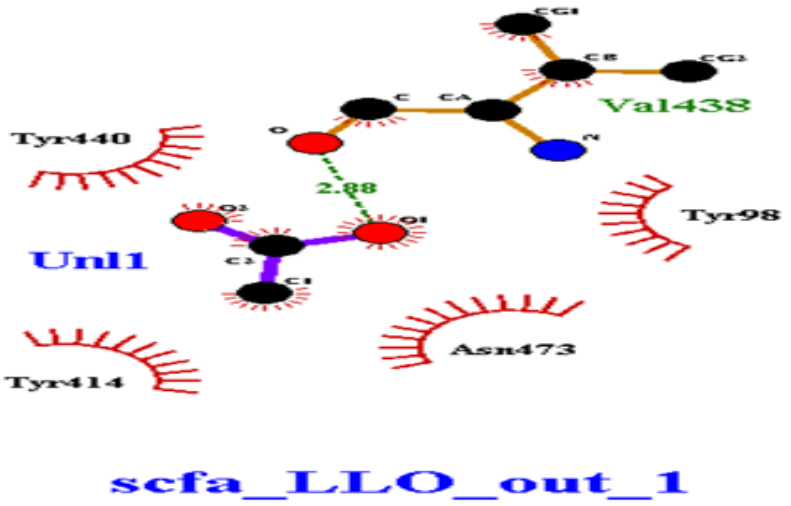


FIGURE 4.27: Interaction of short chain fatty acids with LLO

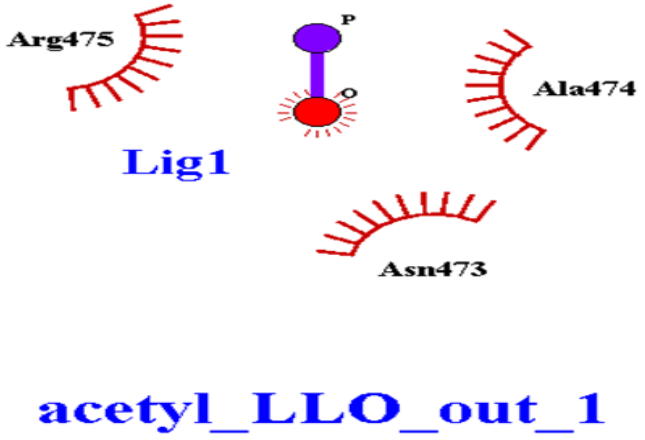


FIGURE 4.28: Interaction of Acetyl phosphate with LLO

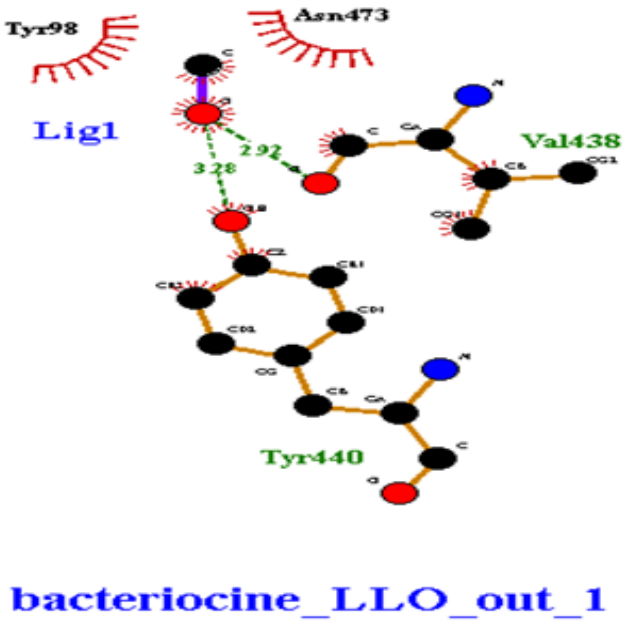


FIGURE 4.29: Interaction of Bacteriocin with LLO

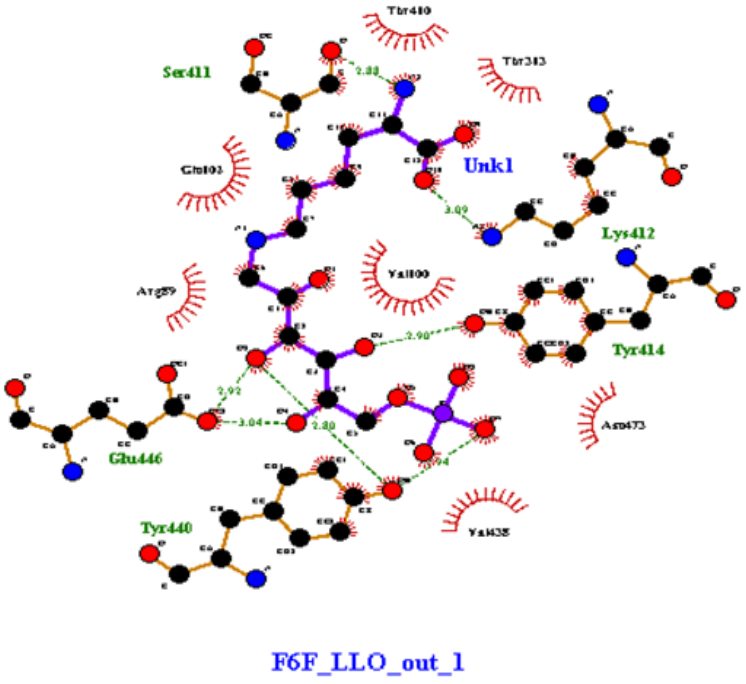


FIGURE 4.30: Interaction of Fructose 6 phosphate with LLO

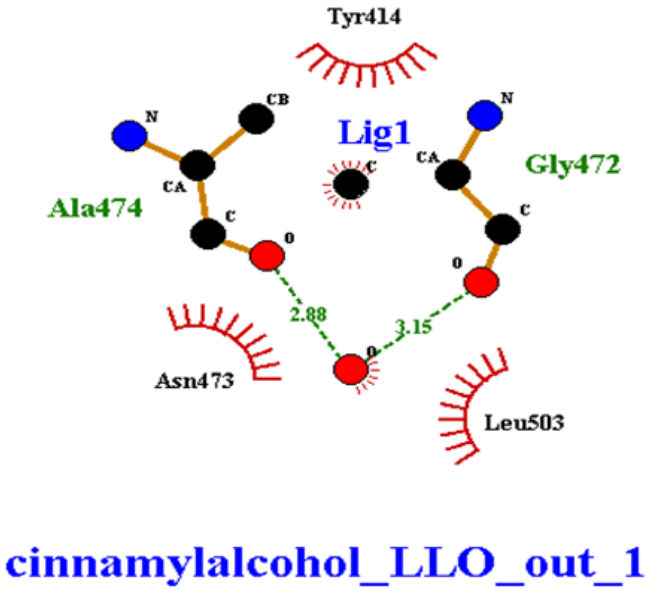


FIGURE 4.31: Interaction of Cinnamyl alcohol with LLO

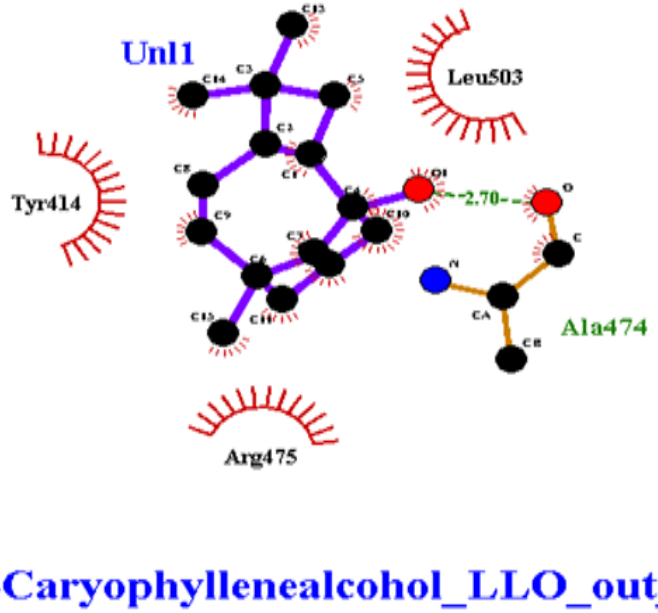


FIGURE 4.32: Interaction of β-Caryophyllene alcohol with LLO

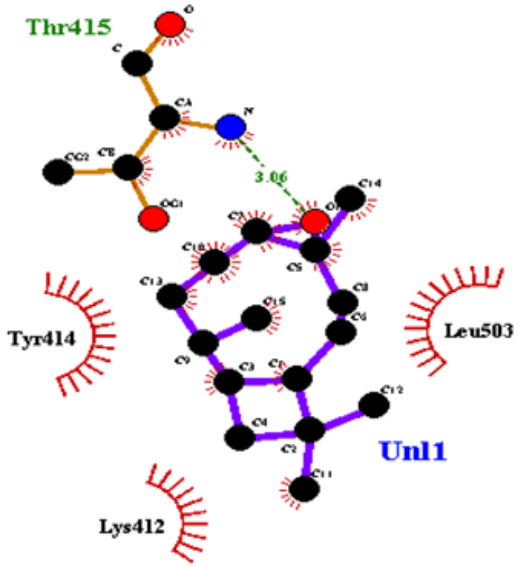


FIGURE 4.33: Interaction of Caryophyllene oxide with LLO

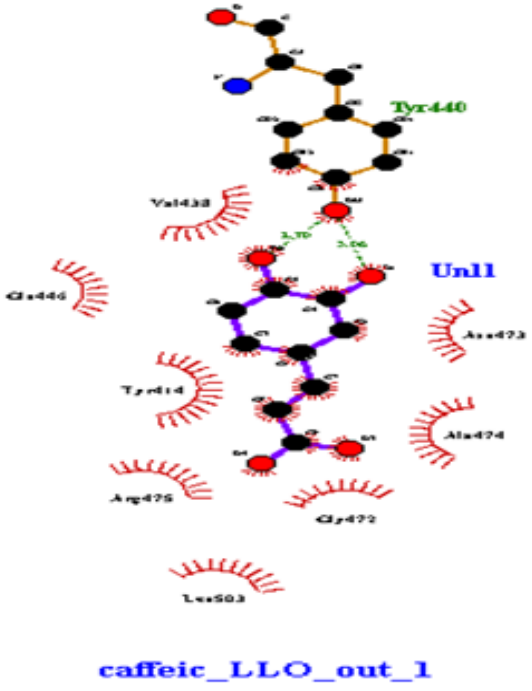


FIGURE 4.34: Interaction of Caffeic acid with LLO

FIGURE 4.35: Interaction of Gallic acid with LLO

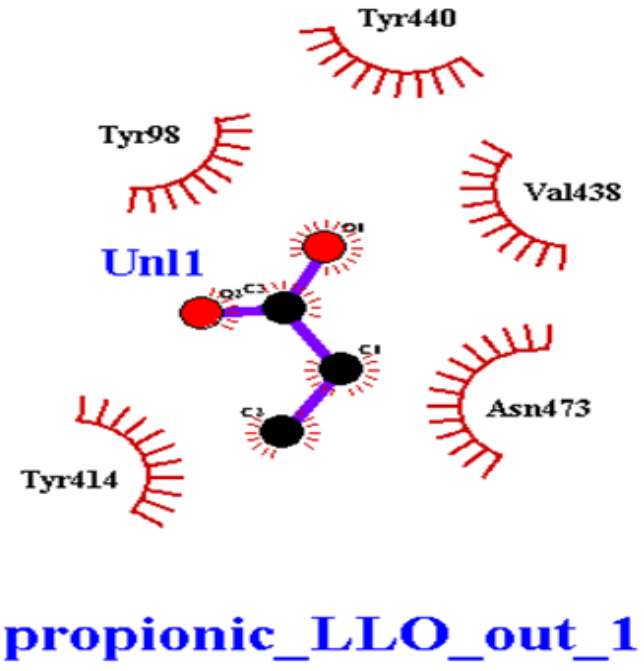


FIGURE 4.36: Interaction of Propionic acid with LLO

Table 4.13: Active Ligand showing Hydrogen and Hydrophobic Interactions with Listeriolysin O.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Short chain fatty acid	-3.4 & 1	Val438	2.88	Tyr98 Tyr414 Tyr440 Asn473
2	Acetyl phosphate	-4.1 & —	—	—	Asn473 Ala474 Arg475
3	Bacteriocins	-6.2 & 2	Val438 Tyr440	2.92 3.28	Tyr98 Asn473
4	Fructose-6-phosphate	-5.6 & 6	Ser411 Lys442 Tyr414 Tyr440 Glu446	2.30 2.33 2.90 2.92 2.94 3.04 3.09	Thr410 Asn473 Thr313 Val100 Val438 Arg59

Continue Table 4.13: Active Ligand showing Hydrogen and Hydrophobic Interactions with Listeriolysin O.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Hydrogen Bonding Distance	Hydrophobic Bonding
5	Cinnamyl alcohol	-5.6 & 2	Gly472 Ala474	2.88 3.15	Tyr414 Asn473 Leu503
6	β-Caryophyllene alcohol	-5.9 & 1	Ala474	2.70	Tyr414 Arg475
7	Caryophyllene oxide	-6.2 & 1	Thr415	3.06	Lys412 Tyr414 Leu503
8	Caffeic acid	-5.9 & 2	Tyr440	2.70 3.06	Leu503 Tyr414 Val438 Gln446 Asn473 Ala474 Leu503 Gly472

Continue Table 4.13: Active Ligand showing Hydrogen and Hydrophobic Interactions with Listeriolysin O.

S.No	Ligands Name	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
9	Gallic acid	-5.4 & 1	Tyr440	3.01	Val100 Tyr414 Val438 Gly472 Asn473 Ala474 Arg475
10	Propionic acid	-3.6 & —	—	—	Tyr98 Tyr414 Val438 Tyr440 Asn473

If we compare the overall hydrogen bonding and hydrophobic interaction of all metabolic compounds, so fructose-6-phosphate and Caffeic acid are only two metabolic compounds which shows more hydrogen bonding and hydrophobic interaction as compared to other compounds.

4.8 ADME Properties of Metabolic Compounds

Lipinski rule of five law used as an initial step for evaluating drug oral bio availability and artificial accessibility. Second step and drug designing screening is assessment of ADME properties of metabolic compounds performed by using online tool pkCSM (pharmacokinetics for small prediction molecules) these onlinetool .gives results by inserting canonical similes of a compounds [122].

4.8.1 Pharmacodynamics

Pharmacodynamics is branch of pharmacology in which we study the biochemical and physiological effects of drug on the body.

4.8.2 Pharmacokinetics

Pharmacokinetics is another broad term used in pharmacological study so this deals with the studies of body mechanism on the drug such as the reaction of body in response to induce drug and its mechanism of action so these broad term contain properties such as absorption, distribution, metabolism and excretion. All the ADME properties are discuss below.

4.8.3 Absorption

In pharmacology absorption is said to be the transfer of drug passes from blood stream into the tissue. Absorption is one of important properties which indicates the absorption of oral administration of drugs so these drugs are generally absorb through of the body [123].

Absorption is one of ADME properties which predict absorption of orally administered drugs and includes Water solubility, Caco2 permeability, Intestinal absorption, Skin permeability, P-glycoprotein substrate, and P- glycoprotein I & II inhibitors. Water solubility (log S) of a compound predicts its solubility in water

Absorption properties contain different parameters like water solubility, caco2 permeability skin permeability, intestinal absorption (human) p glycoprotein substrate, p glycoprotein inhibitor I and II. Water solubility gives metabolic compound values in the form of log mol/l.

So this shows solubility of metabolic compounds in water at 25^oC, hence water soluble drug shows higher solubility level as compare to lipid soluble drugs [123]. Caco2 solubility predict us the logarithm of permeability coefficient appearance. A compound has high permeability ratio if its value is greater than 0.90.

Below Table 4.14 shows the absorption properties of selected ligands taken through PkCSM online ADMET properties prediction tool. Carylophyllene oxide shows less solubility of water, whereas bacteriocins shows less CaCO2 solubility, fructose 6 phosphate shows poor intestinal absorption in humans as compare to other compounds.

Intestinal absorption shows of the value of compound absorbed in the small intestine of human so the normal range value for intestinal absorption is 50% and high range value for intestinal absorption is more than 50%.

Acetyl phosphate and Caryophyllene oxide are only two compounds which shows less skin parmibility ratio. Skin permeability models predict absorbance of drug in the skin with the reference value more than -2.5 having low permeability.

Short chain fatty acid, Bacteriocins and prophonic acid are three compounds which present p glycoprotein substract but only in this compound bacteriocin havi p glycoprotein inhibitor I and the rest of all compounds do not contain p glycoprotein substract and p glycoprotein inhibitor II these both are absent in the remaining compounds.

So the skin permeability model have directly link with p glycoprotein substrate so these substrate contain ABC transporter that extrudes toxins and other chemicals from entering cells by acting has a biological barriers. P glycoprotein inhibitor I and II shows that us there is inhibitors or not.

TABLE 4.14: Absorption properties of selected metabolites.

Sr.no	Ligands	Water Solubility (mol/L)	CaCO2 Solubility (cm/S)	Intestinal Absorption (human)	Skin permeability (log Kp)	P-glyco-protein substrate	P-glyco-protein I inhibitor	P-glyco-protein II inhibitor
1	Short chain-fatty acid	1.052	1.448	100%	-2.718	Yes	No	No
2	Acetyl - phosphate	0.015	0.505	83.783%	-3.175	No	No	No
3	Bacteriocins	-3.712	-0.108	38.841%	-2.745	Yes	Yes	No
4	Fructose-0.485-Phosphate	-6.773	0.135	30.635%	-2.899	No	No	No
5	Cannmyl alcohol	-1.846	1.612	92.669%	-1.702	No	No	No
6	β Cryophyllen-alcohol	-3.993	1.489	93.711%	-2.2858	No	No	No
7	Cyrophyllen oxide	-4.321	1.414	95.669%	-3.061	No	No	No
8	Caffeic acid	-2.33	0.634	69.407%	-2.722	No	No	No
9	Gallic acid	-2.56	-0.081	43.374%	-2.735	No	No	No
10	Propionic acid	0.833	1.439	100%	-2.789	Yes	No	No

4.8.4 Distribution

Distribution is an important parameter in pharmacology which deal with the movement of drug with in body from part of the body to another part. Distribution is the one of adme property which include four properties such as volume distribution in humans (logl/kg) fraction unbound (fu), blood brain barrier (BBB)(logBB) and CNS permeability (logps) [124].

Volume distribution explain the total volume which the drug will need to be evenly distribute to provide same concentration of drug in the blood plasma volume distribution is considered low.Volume of distribution in human (VDss defined as log L/kg) is one of the ADMET properties that contains four models. Fraction unbound in humans (Fu), permeability of the blood-brain barrier (BBB) expressed as log BBB, and permeability of the central nervous system expressed as log PS.

If the value is less than 0.71 lit/kg and if the value is higher than 2.81lit/kg so it can be considered as high volume distribution so it means that the volume distribution is high.

So the drug can be properly distributed from tissue to plasma. Fraction unbounded values shows us if a compound value is more so it means that these compounds are more effective.

Blood brain barrier permeability protect the brain from exogenous chemical or compounds so the blood brain barrier permeability play a vital role in this situation if the value is less than -1.

So it can not damage to the brain but if value the value is greater than 0.3 so it means that the chemical substance can easily cross the blood brain barrier permeability and it can badly damage to the brain.

If a compound CNS permeability value is greater than -2 so it can cause damage to the central nervous system but if value is less than -3 so it can considered as safe compound.

TABLE 4.15: Distributive properties of selected Metabolites.

S.No	Ligands	VDss (human) (L/kg)	Fraction unbound (human)	BBB permeability (log BB)	CNS permeability (log PS)
1	Short chain fatty acid	0.664	0.891	-0.31	-2.56
2	Acetyl Phosphate	0.242	0.807	-0.252	-3.447
3	Bacteriocin	-0.729	0.269	-1.852	-3.764
4	Fructose-6-phosphate	-0.084	0.777	-1.449	-4.283
5	Cinnamyl alcohol	0.295	0.343	0.478	-1.755
6	β/Caryophyllen alcohol	0.581	0.352	0.581	-2.706
7	Caryophyllene oxide	0.564	0.327	0.647	-2.521
8	Caffeic acid	-1.098	0.529	-0.647	-2.608
9	Gallic acid	-1.855	0.617	-1.102	-3.74
10	Propionic acid	-0.756	0.723	-0.756	-2.675

4.8.5 Metabolism

Metabolism is a process in which we can study a breakdown of molecules in the body. Detoxification in the liver is done by in enzyme which is cytochrome p450 so it release the xenobiotics chemicals when it reacts to toxic chemicals. Commonly drugs need to inactivate against this enzyme but some drugs were still active against cytochrome p450 [125]. So during the metabolism process of the drug contain inhibitors of this enzyme which are not use as they affect the metabolism process of the drug. CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 are inhibitors of various isoform of cytochrome p450 enzyme. So this enzyme contain two substrates naming CYP2D6 and CYP3A4. Metabolic property in table 4.16.

Table 4.16: Metabolic properties of selected metabolites.

Sr.no	Ligands	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
1	Short chain fatty acid	No	No	No	No	No	No	No
2	Acetyl phosphate	No	No	No	No	No	No	No
3	Bacteriocin	No	No	No	No	No	No	No
4	Fructos-6-phosphate	No	No	No	No	No	No	No
5	Cinnamyl alcohol	No	Yes	No	No	No	No	No
6	β-Caryophyllen alcohol	No	No	Yes	Yes	Yes	No	No
7	Caryophyllene oxide	No	No	Yes	Yes	Yes	No	No
8	Caffeic acid	No	No	No	No	No	No	No
9	Gallic acid	No	No	No	No	No	No	No
10	Propionic acid	No	No	No	No	No	No	No

4.8.6 Excretion

Excretion of drug is mainly perform by two organs which is kidney and liver and the rest of other organs like lungs can also take part in excretion by eliminating gaseous and volatile substances. Sweating of the body and salivary gland can also take part excretion of drug [126]. Renal oct2 substrate transporter that clear the drug and other compounds and then excrete through kidney. Renal oct2 clearance shows the excretion value of drug and total clearance shows us liver clearance which means the drug is metabolite. Model of excretory properties a total clearance which unit are ml/kg and other properties are renal oct2 substrates which predict results in the form of Yes or No. Excretory properties of ligands are listed in tables 4.17 below.

Table 4.17: Excretory properties of selected metabolites.

S No	Ligands	Total Clearance	Renal OCT2
1	Short chain fatty acid	1.016 ml/Kg	No
2	Acetyl phosphate	0.168 ml/Kg	No
3	Bacteriocin	0.283 ml/Kg	No
4	Fructose-6-phoaphate	0.529 ml/Kg	No
5	Cinnamyl alcohol	0.253 ml/Kg	No
6	β-Cyrophyllen alcohol	0.74 ml/Kg	No
7	Cyrophyllene oxide	0.905 ml/Kg	No
8	Caeffic acid	0.508 ml/Kg	No
9	Gallic acid	0.518 ml/Kg	No
10	Propionic acid	0.396 ml/Kg	No

4.9 Lead Compound Identification

Identification of lead compound is based on different parameters such as physico-chemical properties, ADMET properties, Lipinski rule of five, docking scores and

interaction properties, so if the drug follow all these properties so it can be considered as lead compound. Physicochemical properties and Lipinski rule of five works as primary filter and admet properties studies has a secondary filter in the screening of lead compound [127]. All metabolic compounds which are selected for Insilico study were obey Lipinski rule of five so these compounds having molecular weight less than 500, logp value also less than 5, hydrogen bond acceptor of these compounds having less than 10 and hydrogen bond of all compounds (short chain fatty acid Bacteriocins, Acetyl phosphate, Cinnamyl alcohol, β-caryophyllene Alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid and Propionic acid) should be less than 5 except fructose-6-phosphate.

Admet properties study of these compounds such as short chain fatty acid Bacteriocins, Acetylphosphate, Cinnamylalcohol, β-caryophyllene Alcohol, Caryophyllene oxide, Caffeic acid, Gallic acid, fructose-6-phosphate and Propionic acid were screen out. To check all parameters like physciochemical properties, admet properties, Lipinski rule of five interaction properties and docking score we identified lead compound which is Caryophyllene oxide so this compound contain all properties which are required for lead compound. Caryophyllene oxide is our lead compound for this Insilco study.

4.10 Drug Identification Against Listeriosis

The US food and drug administration (FDA) approved medicine for the treat of listeriosis disease. Ampicillin and gentamicin both are antibiotics which is mostly used to treat bacterial infection like gram positive and gram negative infection. Almost these drugs (GENTAMICIN AND AMPICILLIN) have been used throughout the world specifically in Pakistan, UK, Brazil and India.

The docking results of ten compounds were compare with two FDA approved drugs namely ampicillin and gentamicin [128]. There 3D structure were download from obtain database in sdf format and 3D optimization and energy minimization through Chem 3D pro (version 12.0)

TABLE 4.18: Reference drugs with its mechanism of action.

S.No	Drugs	Mechanism of action	References
1	Gentamicin	The entrance of aminoglycosides into cells occurs in three distinct stages. The first ionic binding phase starts when polycationic aminoglycosides electrostatically bind to negatively charged bacterial cell membrane components, such as the lipopolysaccharides and phospholipids in Gram-negative bacteria's outer membrane and the teichoic acids and phospholipids in Gram-positive bacteria's cell membrane.	[129]
2	Ampicilin	Ampicillin prevents the third and final stage of bacterial cell wall formation by interacting with particular penicillin-binding proteins (PBPs) found inside the bacterial cell wall. Then, bacterial cell wall autolytic enzymes like autolysins, which are responsible for cell lysis; it's likely that ampicillin interacts with an autolysin inhibitor.	[130]

4.10.1 Gentamicin

Gentamicin is a bactericidal aminoglycoside discovered and isolated in 1963 from Micromonospora purpurea. Because of its broad spectrum of activity, low cost, and wide availability, it is one of the most commonly prescribed aminoglycosides. Gentamicin eliminates both gram-positive and gram-negative bacteria. Gentamicin is FDA approved drug which is mostly used to relief the symptoms of mild to severe bacterial infections. There are several distinct antibiotics in the aminoglycoside class. Gentamicin has been approved by the US Food and Drug Administration (FDA) for clinical use [131]. So there is different databases available to retrieve FDA approved drugs like gentamicin. Drug bank and PubChem is freely accessible online database from which we can easily retrieve FDA approved drug with its

mechanism of action. Gentamicin is an antibiotics which is most probably used to treat bacterial infection. So the 2D structure of gentamicin is retrieve from PubChem database.

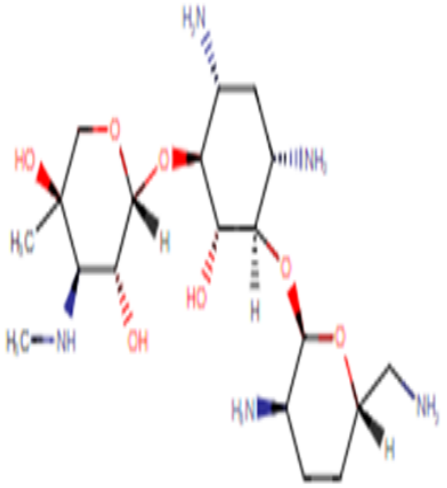


FIGURE 4.37: 2D structure of Gentamicin retrieve from PubChem database.

4.10.2 Gentamicin Effects on the Body

The most common side effects gentamicin are that occurs nausea, vomiting diarrhea , headache, weight loss, troubling in sleeping, muscles cramps and dizziness. Long term use of this drug could lead to allergic reaction and also lead to swelling of face infection [132].

4.11 ADMET Properties of Identified Drug

The ADMET properties of identified drugs are studies using the same software which is easily available database so the software which are most frequently used were pkCSM software for ADMET properties identification.

4.11.1 Toxicity prediction of reference drug

The toxicity prediction is an important parameter to check the toxicity of a drug. So this toxicity parameters consist of maximum tolerated dose value which is less than -0.098 , second parameter contains two parameter naming herg I AND Herg II inhibitors, so these inhibitors mean that it can inhibit potassium channel. Acute and chronic toxicity shows us the minor and major toxicity rate. Hepatotoxicity of a drug is high so it can cause liver problems, gentamicin shows high rate of chronic toxicity it means that it can cause major chronic toxicity so it we compare acute toxicity with chronic toxicity so the chronic toxicity rate is higher. Tpyriformis toxicity and minnow toxicity shows us the toxic compound of end point. If we compare both toxicity value so the minnow toxicity vale is greater than Tpyriformis toxicity. Minnow toxicity predicts lc50 in millimeter which represent the lethal concentration of a molecule sufficient to cause a deaths of 50%. Gentamicin predicit mennow toxicity value has 6.242m/m

4.11.2 Absorption properties of reference drug

Gentamicin shows absorption properties which are shown in the following table. So from the given table it is clear that gentamicin is less soluble in water and its intestinal absorption is less than 50% so it means that this drug is not properly absorbed in human intestine. Skin permeability is low and shows positive results as P glycoprotein substrate it means that the reference drug has low oral absorption. P glycoprotein I and II inhibitors "No" means that gentamicin has no pumping activity to pump out xenobiotic from cells.

4.11.3 Distribution properties of reference drug

Distribution properties consist of four models, among these model the first one is volume distribution in human (logl/kg). Gentamicin shows low volume distribution which means that gentamicin is not properly distributed in tissue to

plasma. fraction unbounded means that that this drug is unbound to plasma if it unbounded fraction is more than these drug may be more effective. Our reference drug has 0.744 fraction unbounded predicted value. Blood brain barrier permeability express as logBB unit, shows value of -0.851 is greater than -1 and considered as safe to not cross brain barrier permeability.

Last model naming CNS permeability express as log ps greater than -3 but if we look at CNS permeability value of gentamicin which is less than -3 so the actual value of CNS permeability is -4.093. The distribution properties of reference drug is given in the below table.

4.11.4 Metabolic properties of reference drug

Reference drug metabolic properties are discussed in the given table. Cytochrome p450 is detoxification enzyme present in liver which can play a vital role in excretion of exogenous compound.

CYP2D6 and CYP3A4 are two main substrate of cytochrome p450. First and second model shows that gentamicin is not metabolized by cytochrome p450. All inhibitors shows that drug is not an inhibitory for cytochrome p450.

4.11.5 Excretory properties of reference drug

Excretion properties consist of two model and their values are given in the below table. Total clearance value is 0.708ml/kg which indicates the hepatic and renal clearance of gentamicin.

Renal oct2 substrate is an organic cation transporter having role in disposition and renal clearance of drugs. Gentamicin predicts renal oct2 substrate "NO" which mean that it is not interfering in the function of oct2 in the cells.

Being oct2 substrate has harmful effects when react with inhibitors. All the ADMET properties of reference drug were shown in Table 4.19.

Table 4.19: ADMET properties of reference drug gentamicin.

S No	ADMET Properties	Model Name	Gentamicin
01	**Toxicity**	AMES toxicity	No
		Max. tolerated dose (human)	0.188 mg/kg
		hERG I inhibitor	No
		hERG II inhibitor	No
		Oral rat acute toxicity	2.559 mol/kg
		Oral rat chronic toxicity	2.763 mg/kg
		Hepatotoxicity	No
		Skin sensitization	No
		T. pyriformis toxicity	0.285 log ug/L
		Minnow toxicity	6.242 log Mm.
02	**Absorption**	Water solubility	-2.843 mol/L
		CaCO2 solubility	0.979 cm/S
		Intestinal Absorption (human)	19.161%
		Skin permeability	-2.735 log Kp
		P-glycoprotein substrate	Yes
		P-glycoprotein I inhibitor	No
		P-glycoprotein II inhibitor	No
03	**Distribution**	-VDss (human)	-1.313 L/kg
		Fraction unbound (human)	0.744 Fu
		BBB permeability	-0.851 log BB
		CNS permeability	-4.093 log PS
04	**Metabolosim**	CYP2D6 substrate	No
		CYP3A4 substrate	No
		CYP1A2 inhibitor	No

		CYP2C19 inhibitor	No
		CYP2C9 inhibitor	No
		CYP2D6 inhibitor	No
		CYP3A4 inhibitor	No
05	**Excretion**	Total Clearance	0.708 ml/kg
		Renal OCT2 Substrate	No

4.11.6 Physicochemical properties of reference drug

Physicochemical properties shows the basic and fundamental characteristics of compounds these properties also work as a primary screener. The molecular weight of gentamicin is 477.603, logp value of gentamicin is -3.3275 whereas the hydrogen bond donor of reference drug is 8 while hydrogen bond acceptor value is 12. If we look at hydrogen bond donor and acceptor value so these value is greater than its normal value and this drug cannot follow Lipinski rule properly.

Table 4.20: Physiochemical properties of reference drug

S.No	Physicochemical properties	Predicted values of Gentamicin
1	Molecular weight	477.603 g/mol
2	Log p value	-3.3275
3	Hydrogen bond acceptor	12
4	Hydrogen bond donor	8

4.11.7 Molecular docking of reference drug

Gentamicin is a ligand which dock with three different proteins naming Internalin A, Internalin B and Listeriolysin O. best docking score was -6.6 with Internalin A receptors. Molecular docking process were done by CB dock online software which gives results in five best poses with cavity size and calculating its size and center

value as grid map values. This ligand shows poor binding score with Internalin B and Listeriolysin O.

TABLE 4.21: Molecular docking of reference drug with different proteins.

S.No	Parameters	Gentamicin with Internalin A	Gentamicin with Internalin B	Gentamicin Listeriolysin O
1	Binding score	-6.6	-6	-6
2	Molecular weight	477.603	477.603	477.603
3	Logp value	-3.3275	-3.3275	-3.3275
4	HBA	12	12	12
5	HBD	8	8	8
6	Cavity size	2017	131	983
7	Min energy kcal/mol	-4.3539	-4.3539	-4.3539
8	Max energy kcal/mol	38.3608	38.3608	38.3608

4.12 Physiochemical properties comparison of reference drug and lead compound

The reference drug and lead compound were compare on the basis of their physicochemical properties as well as admet properties, docking score comparison and interaction properties, so these properties assess their bio availability, drug likeness, efficacy and safety. if we compare the lipikinsi rule of five likeness criteria so gentamicin fail Lipinski rule f five criteria while on the other hand Caryophyllene oxide compound passed the drug likeness criteria [133]. However Caryophyllene oxide showing less molecular weight, logp value hydrogen bond acceptor and hydrogen bond donor value from its normal range value. But if we compare hydrogen bond acceptor and hydrogen bond donor value of gentamicin which normal range

value so its value is greater than normal range value that's why our lead compound is best as compare to gentamicin on the basis of Lipinski rule of five. Lipinski rule of five are given below.

1. Hydrogen bond donor should be less than 5.
2. Hydrogen bond acceptor should be less than 10.
3. Molecular weight should be less than 500.
4. Log p value of molecule should be limited to 5.

Table 4.22: Physiochemical properties comparison of reference drug and lead compound.

S.No	Parameters	Gentamicin	Caryophyllene oxide
1	Molecular Weight	477.603 g/mol	220.326 g/mol
2	Log P Value	-3.3275	3.9364
3	HBD	12	1
3	HBA	8	0

4.13 Admet properties comparison of both reference drug and lead compound

Admet properties include absorption, distribution, metabolism, excretion and toxicity parameters which play vital role in screening of compounds as drug candidates. Pharmacokinetics properties of reference drug and lead compound were listed below.

4.13.1 Absorption properties comparison of reference drug and lead compound

Water solubility of referance drug is more than lead compound. Caco2 permeability shows us about absorption of orally administred drug. Predict values of

caco2 model with in safe range for lead compound Caryophyllene oxide shows better value than gentamicin. Intestinal absorption in human model predict the absorption of drug in the intestine so the predicted value of caryophyllene oxide is 95.669% and for gentamicin the intestinal absorption value is 19.161%. gentamicin falls " Yes" catageory for p-glycoprotein substrate and "NO" category of p-glycoprotein I and II inhibitors while Caryophyllene oxide fall in "NO" category for all three models. This mean that gentamicin has p-glycoprotein substrate shows low oral absorption.

TABLE 4.23: Absorption properties comparison of reference drug and lead compound.

S.No	**Model Name**	**Predicted Values**	
		Gentamicin	**Caryophyllene oxide**
1	Water solubility	-2.843	-4.321
2	Caco2 permeability	0.979	1.414
3	Intestinal absorption	19.161%	95.669%
4	Skin permeability	-2.735	-3.061
5	P-glycoprotein substrate	Yes	No
6	P-glycoprotein I inhibitor	No	No
7	P-glycoprotein II inhibitor	No	No

4.13.2 Distribution properties comparison of reference drug and lead compound

First model of distribution properties is volume distribution which predicts low value for gentamicin and high value for Caryophyllene oxide. Volume distribution low value consider unsafe because high value indicates the drug mostly distributed in the tissue than plasma. Fraction unbound value of Caryophyllene oxide. Fraction unbounded distribution value of gentamicin is more than Caryophyllene oxide which shows that gentamicin is not more effective due to at high fraction unbounded distribution value. Blood brain barrier permeability greater than 2 which means that the drug harm to brain and these drug can easily cross blood brain barrier permeability.our lead compound Caryophyllene oxide shows less blood brain

barrier permeability value than normal range value so it means that this compound can not harm to brain and not easily cross blood brain barrier permeability. CNS permeability of a drug is greater than -3 so it means that the drug is considered as safer. If we compare both values of CNS permeability of reference drug and lead compound so our lead compound is much better as compared to reference drug. Distribution properties of lead compound and reference drug is given below in the following table.

TABLE 4.24: Distribution properties comparison of reference drug and lead compound.

S.No	**Model Name**	**Predicted Values**	
		Gentamicin	**Caryophyllene oxide**
1	Volume distributions (human)	-1.313	0.564
2	Fraction unbounded (human)	0.744	0.327
3	BBB permeability	-0.851	0.647
4	CNS permeability	-4.093	-2.521

4.13.3 Metabolic properties comparison of reference drug and lead compound

Metabolic properties are predicted on the basis of cytochrome p450 isoforms which are CYP2D6,CYP3A4,CYP1A2, CYP2C19 CYP2C9,CYP2D6 and CYP3A4 of gentamicin did not shows it self has a substrate and inhibitors isoform of cytochrome p450 whereas Caryophyllene oxide shows itself has a three inhibitors isoform of cytochrome p450 inhibitors naming CYP1A2, CYP2C19 and CYP2C9 and it did not shows itself has a substrate isoform of cytochrome p450. Caryophyllene oxide predict itself as inhibitor of CYP2C9 which is a main inhibitor for drug metabolism. Gentamicin did not shows itself as inhibitor isoform. If we compare both values of Caryophyllene oxide and gentamicin so Caryophyllene oxide shows the inhibitory properties over gentamicin.

Table 4.25: Metabolic properties comparison of reference drug and lead compound.

S.No	Model Name	Predicted Values	
		Gentamicin	**Caryophyllene oxide**
1	CYP2D6 substrate	No	No
2	CYP3A4 substrate	No	No
3	CYP1A2 inhibitor	No	Yes
4	CYP2C19 inhibitor	No	Yes
5	CYP2C9 inhibitor	No	Yes
6	CYP2D6 inhibitor	No	No
7	CYP3A4 inhibitor	No	No

4.13.4 Execration properties comparison of reference drug and lead compound

Excretion properties consist of two models naming total clearance and renal oct2 substrate with predicted values are shown in the given table.Drug clearance is measure by total clearance which occur as combination of renal clearance and hepatic clearance. Predicted value of drug clearance as a total clearance of Caryophyllene oxide value 0.905 is higher as compared to gentamicin value 0.708 .

If we compare both clearance value so it means that Caryophyllene oxide total clearance value is much better as compared to gentamicin total clearance value. Both compounds stands in "NO" category for renal oct2 substrate model. Which means that these compound not in the interfering in the normal function of organic cation transporter.

Table 4.26: Excretory properties comparison of reference drug and lead compound

S.No	Model Name	Predicted Values	
		Gentamicin	**Caryophyllene oxide**
1	Total Clearance	0.708	0.905
2	Renal Oct2 substrate	No	No

4.13.5 Toxicity prediction comparison of reference drug and lead compound

Toxicity prediction is most important parameter which consists of nine models. Maximum tolerated dose help to set maximum recommended tolerated dose which shows positive values of gentamicin value is 0.188 log mg /kg and Caryophyllene oxide also shows maximum tolerated dose in positive values which is 0.148 log mg/kg. The model hergI and II inhibitors predicts about either analyzed compounds are inhibitors of potassium channels or not. So if answer is ''NO" than compound may be fully fit for drug. The model oral rat acute toxicity that can cause minnow toxicity about 50% in rats.

Acute rat toxicity of gentamicin is slightly higher than Caryophyllene oxide the value for gentamicin is 2.559 which is greater than Caryophyllene oxide value which is 1.548, it means that gentamicin shows high acute rat toxicity than Caryophyllene oxide. Oral rat chronic toxicity determine the higher dose of drug which produce adverse effects for longer period of time. If we compare both oral chronic toxicity values of gentamicin and Caryophyllene oxide so the chronic toxicity value of gentamicin is 2.763 which is higher than the Caryophyllene oxide value 1.22.

Caryophyllene oxide shows lowest value of chronic toxicity over gentamicin so Caryophyllene oxide having no adverse effect for longer period of time because its chronic toxicity values is lower than gentamicin chronic toxicity value [133]. Hepatotoxicity simply indicates injury to liver so both compounds shows NO hepatotoxicity.. tpyroformis toxicity value predicts ofgentamicin is lower than Caryophyllene oxide.

Minnow toxicity shows lethal toxicity of a conmpound which is necessary to cause deaths because this toxicity is very lethal so the minnow toxicity of reference drug gentamicin is higher as compared lead compound Caryophyllene oxide. Minnow toxicity value for gentamicin is 6.242 and for Caryophyllene oxide is 0.955. minnow toxicity value of gentamicin is higher as compared to Caryophyllene oxide so the Caryophyllene oxide is considered to be safe compound than gentamicin.

TABLE 4.27: Toxicity prediction comparison of reference drug and lead compound.

S.No	Model Name	Predicted Values	
		Gentamicin	**Caryophyllene oxide**
1	AMES toxicity	No	No
2	Max.tolerated dose(human)	0.188	0.148
2	hERG I inhibitor	No	No
3	hERG II inhibitor	No	No
4	Oral rat acute toxicity	2.559	1.548
5	Oral rat chronic toxicity	2.763	1.224
6	Hepatoxicity	No	No
7	Skin sensitization	No	Yes
8	t.pyriformis toxicity	0.285	1.079
9	Minnow toxicity	6.242	0.955

4.14 Docking Scores Comparison of Reference Drug and Lead Compound

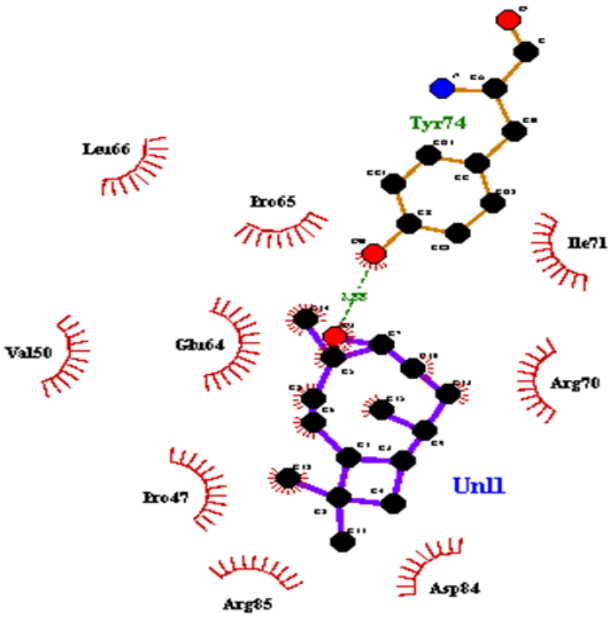
Discovering of new drugs are particularly important in computer aided drug designing. Therefore reference drug as a ligand were docked against selected receptors by CB dock online tool which predicts the binding and cavity sites of protein and calculates center and size of best five poses for all the three proteins separately. Final results of docking of reference drug and lead compound against selected three proteins namely Internalin A, Internalin B, listerolysin O. the highest binding score is -6.6 against Internalin A receptor shows by gentamicin and Caryophyllene oxide binding score against Listeriolysin O is -6.2.

The lowest binding score of Caryophyllene oxide against Internalin A -5.8 and against Internalin B IS -4.9. Gentamicin binding score against Internalin B is -6 and against Listeriolysin O is -6. Maximum energy Caryophyllene oxide against three receptors showing best and strong maximum energy which is 87.9156 kcal/-mol, on the other hand gentamicin shows weak and lower maximum energy which is 38.3608 kcal/mol. Minimum energy of Caryophyllene oxide is also better than gentamicin so the minimum energy for Caryophyllene oxide is -1.2779 kcal/mol,

and minimum energy for gentamicin is -4.3539 kcal/mol. So after minimize energy of Caryophyllene oxide against Internalin A, Internalin B and Listeriolysin O shows strong interaction with three proteins. Reference drug Gentamicin showing very poor interaction against Internalin A, Internalin B and Listeriolysin O [134]. the docking score of gentamicin is little bit high than Caryophyllene but if we compare its interaction properties so gentamicin shows very poor interaction with targeted proteins and if we compare the interaction results of Caryophyllene oxides it is much better than reference drug gentamicin interaction.

TABLE 4.28: Docking scores comparison of reference drug and lead compound.

S.No	Parameters	Binding score with Internalin A	Binding score with Internalin B	Binding score with Listeriolysin O
1	Caryophyllene oxide	-5.8	-4.9	-6.2
2	Gentamicin	-6.6	-6	-6



Caryophylleneoxide_internelinA_out_1

FIGURE 4.38: Interaction of lead compound with Internalin A

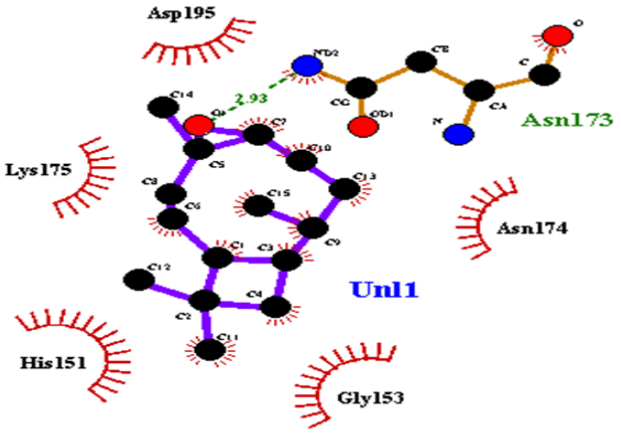


FIGURE 4.39: Interaction of lead compound with Internalin B

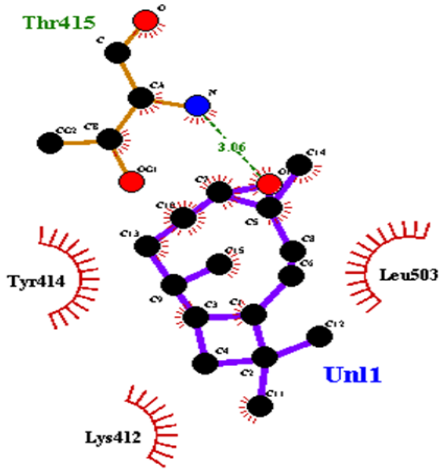


FIGURE 4.40: Interaction of lead compound with LLO

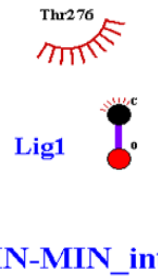
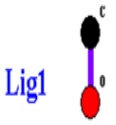
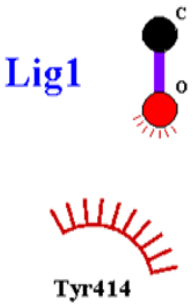


FIGURE 4.41: Interaction of Reference drug with Internalin A.



GENTAMICIN-MIN_internelinB_out_1

FIGURE 4.42: Interaction of Reference drug with Internalin B.



GENTAMICIN-MIN_LLO_out_1

FIGURE 4.43: Interaction of Reference drug with LLO.

The detail of hydrogen bonding and hydrophobic interaction are given in the following table. Oxygen atoms present in ligands which play a vital role in formation of hydrogen bond with target protein. Although Caryophyllene oxide shows more hydrogen bonding and hydrophobic interaction than gentamicin. Interacting amino acids are more in lead compound than reference drug. Furthermore hydrophobic interaction in lead compound are strong in and more in number than gentamicin.

TABLE 4.29: Lead compound and reference drug showing hydrogen and hydrophobic interactions.

S.No	Compound	Binding Energy & No of HBs	Hydrogen Bonding Amino Acids	Distance	Hydrophobic Bonding
1	Caryophyllene oxide	-6.2 & 1	Thr415	3.06	Tyr414 leu503
		-5.8 & 1	Tyr74	2.33	Pro47 Val50 Arg85 Asp80
		-4.9 & 1	Asn173	2.93	Asn174 Gly153 Asp195
2	Gentamicin	-6.2 & — -6 & — -6 & —	— — —	— — —	Tyr414 Thr276 —

Caryophyllene oxide which was identified as a lead compound was not isolated from different species of Bifidobacterium. Because our focus is on novel specie of Bifidobacterium so all metabolites including Caryophyllene oxide were identified from the novel specie of Bifidobacterium. Mostly gentamicin is used as a synthetic drug to treat bacterial infection so on the basis of this property we select gentamicin as reference drug.

Chapter 5

Conclusions and Recommendations

The motive of this research work is to design potential antibacterial compounds from bifidobacterium *aquikifir* and its metabolites to treat listeriosis disease. Ten metabolic compounds which represents almost all class of bifidobacterium *aquikifir* metabolites are selected from literature and databases. The proteins used for virtual screening were Internalin A, Internalin B and Listeriolysin O proteins. Molecular docking is performed by using CB dock online tool against short chain fatty acids, Acetyl alcohol, Bacteriocins, fructose-6-phosphate, Cinnamyl alcohol, beta-caryophyllene alcohol, Caryophyllene oxide- Caffeic acid, Gallic acid and propionic acid are identified as a metabolic compounds. Drug likeness of a compounds are studied and reported by using Lipinski rule of five as a primary and ADMET properties as a secondary filters. Caryophyllene oxides is identified as a lead compound on the basis of its molecular docking results physciochemical properties, Lipinski rule of five, ADMET properties and interaction properties of this compound is compared with FDA approves drug namely gentamicin. Caryophyllene oxide having the potential of binding with target protein more efficiently and shows less toxicity rate than the reference drug. All the software and tool used in current research work are reliable and authentic.

5.1 Recommendations

Lead compound "Caryophyllene oxide" as per this research studies and results should be explored as a drug candidate for the treatment of listeriosis neonatal disease. More research is needed to explore the exact mechanism of action as well as the impact on human body and safety concerns. Caryophyllene oxide shows antibacterial, anti-inflammatory and pharmacological effects making it interesting and important to investigate the medical effects and molecular mechanism using modern disease pathophysiological concept. constant observation and basic research on bacterial virulence and intracellular signaling will continue to promote the development of new and effective medicines. So from the current research work we identified that Caryophyllene oxide in future can act as efficient and effective drug for the treatment of listeriosis disease.

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