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Preliminary *in silico* study of a novel paratransgenic weapon against malaria: Genetically modifying wild *plasmodium* populations *via* recombinant mosquito symbiont

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ABSTRACT

Malaria is an infectious disease caused by *Plasmodium spp.*, a protist whose infection is spread by *Anopheles* mosquito as a vector. A potential method to counter the infection is through paratransgenesis, a promising genetic control approach. This study proposed a new approach by using a transgene carried in a modified Ti plasmid hosted in *Asaia* bacterium to carry out two purposes: sensing the presence of *Plasmodium* protein biomarkers and transferring the toxin transgene to the parasite upon detection, thereby killing it without harming the *Asaia* carrier. The biosensor mechanism was created by repurposing a TonB dependent iron-uptake transport pathway to transcribe the *vir* genes of *Agrobacterium tumefaciens* that facilitate the gene transfer. The aim of this *in silico* project was to serve as a preliminary study on the likelihood of success of the aforementioned biosensor mechanism. This study utilized Alphafold and RCSB (Research Collaboratory for Structural Bioinformatic Protein Data Bank). As for the research tools: PyMOL, ClusPro website, and PRODIGY (PROtein binDIng enerGY prediction) server was used for data preparation, protein-protein docking and binding affinity analysis respectively. The results were assessed with t-test to analyze the significance of the binding affinity, in comparison to other studies that employed similar methods. The result showed *Plasmodium* protein GGCS (Gamma-glutamylcysteine synthetase) having the highest binding affinity with FecA (Fe(3+) dicitrate transport protein A). The preliminary data suggested that introducing the toxin transgene may be possible through *vir* gene transcription from the TonB pathway.

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1. INTRODUCTION

Indonesia had the second largest number of malaria cases in Asia after India, according to the 2022 data from World Health Organization [1]. In 2021, the estimated cases of Malaria in Indonesia were 811,636 positive cases [1]. There has been an increasing trend of malaria cases in Indonesia, where most positive cases are found in the eastern region of the country, especially in Papua, West Papua, Maluku, and East Nusa Tenggara provinces [1]. Nearly 89% of malaria cases are still reoccurring in these areas.

The female *Anopheles* mosquitoes are infamous for their inadvertent role in spreading malaria [2]. The symptoms of malaria include fever, fatigue, rapid heart rate, and coughing in milder cases and diarrhea, myalgia, and anemia in severe ones. This disease may also lead to death in the most critical cases [3]. Female *Anopheles* mosquitoes require proteins within mammalian blood, including humans, to facilitate the maturation of their eggs [4]. However, *Plasmodium* parasites exploit this maternal behavior by using the mosquito as a host for their reproduction. These parasites multiply within the mosquito, reaching significant numbers. When the *Plasmodium*-infected mosquito subsequently feeds on an uninfected human, the parasites are transmitted to the human host, where they continue to reproduce [5]. In the human host, they remain dormant until another mosquito bite restarts the cycle [6], [7]. Six *Plasmodium* species known to cause malaria in humans: *P. falciparum*, *P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri,* and *P. knowlesi* [8].

Current approaches in addressing this disease may have not been sufficient. For example, the most widely available antimalarial vaccine at the moment is the RTS,S/AS01 vaccine (trade name Mosquirix), targeting *P. falciparum*. However, it falls short in providing a complete immunity [9]. This vaccine primarily focuses on the circumsporozoite protein, a crucial component in the *Plasmodium* life cycle [10]. The challenge, however, lies in the fact that the protein is polymorphic in nature [11]. Therefore it is hard to develop a standardized vaccine that works against all types of polymorphism of this protein. Moreover, even when dealing with vaccine-matching malaria, the efficacy of RTS,S/AS01 starts at approximately 50-60% and gradually decreases over time [9]. When efforts to directly control *Plasmodium* parasites prove ineffective, another approach is to target their insect carriers, the *Anopheles* mosquitoes, by utilizing insecticides [12]. However, it's important to recognize that insecticides offer only a temporary solution. Once the application of insecticides is discontinued, the mosquito population tends to rebound, making a periodic application necessary. This method is also non-sustainable and temporary [13], [14]. A repeating application of insecticides can also lead to the development of resistance within mosquito populations [15].

Genetic engineering approach, on the other hand, offers several advantages, including a lifetime retaining of the genetic modification in the host which can be shared vertically with its offspring in transgenesis [16] or horizontally among other living hosts in paratransgenesis [13]. Moreover, this approach also has high specificity which can avoid affecting non targeted organisms [17]. Genetic engineering can be done on the mosquito vectors to hamper the life cycle of *Plasmodium* [18] or to spread infertility and cull mosquito populations among several generations [18]. This approach is known as transgenesis. However, another genetic modification that can be done to the microbial symbiont living with and within the mosquito can also be an option. This kind of engineering may help eliminate the pathogenic *Plasmodium* parasites. This kind of genetic engineering is known as paratransgenesis [19] that offers several advantages over conventional transgenesis. First, it is often more cost-effective and simpler to genetically modify smaller, single-celled microbial organisms compared to large, multicellular organisms like mosquitoes [20]. Second, the genetic modifications made to the microbial symbionts can be vertically transmitted to all of their offspring when their cells replicate within the mosquito, leading to an exponential amplification of the modification's impact based on the symbiont population size. In contrast to transgenesis, which typically only affects a single mosquito species, a genetically modified microbial symbiont has the potential to be effective across a wide range of mosquito species [13]. These advantages make paratransgenesis an appealing approach for this research project [21]. In this *in sillico* study, the design for mitigation of *Plasmodium* parasite utilizes an unconventional effector molecule: a toxin gene. This toxin gene is expected to be continuously expressed within the *Plasmodium* cell, leading to the constant production of toxin molecules and eventual demise of the parasite. However, the origin of the toxin gene must be properly considered. An engineered microbial symbiont, specifically the bacterial genus *Asaia*, would harbor the toxin gene in an inactive state, housed in a plasmid. The Ti (tumor inducing) plasmid of *Agrobacterium tumefaciens* was chosen due to its inherent gene transfer mechanism. The logic is that when in close proximity to a *Plasmodium* cell, *Asaia* would transfer the toxin gene to the parasite.

Subsequently, the gene would be expressed, toxin production initiated, and the parasite can be eliminated. Additional intricacies are elucidated in the subsequent sections below.

This study scope was focused on an initial *in silico* examination aimed at identifying potential *Plasmodium* biomarkers that exhibit compatibility with the gene transfer mechanism in the Ti plasmid. The identified proteins of interest served as initial targets for subsequent investigation that must be conducted *in vitro* in the future. The selection of an appropriate symbiont to host this genetic modification is crucial to ensure the desired impact within the mosquito. Various desirable traits must be taken into account when considering a host symbiont. Preferably, the symbiont should exhibit non pathogenicity to humans, thereby excluding species with a documented biohazardous history. Furthermore, the symbiont should possess the capability to colonize multiple mosquito species, all of which serve as vectors for malaria, ensuring the efficacy of the genetic control method. The selected host symbiont should also thrive within the mosquito's gut environment, facilitating the substantial expression of effector molecules for optimal effectiveness. Microbes indigenous to the mosquito microbiota emerged as the best prime candidates in this regard [13] with a substantial array of symbionts has been documented to date. Notable examples within the indigenous microbiota of *Anopheles* mosquitoes include *Enterobacter spp*. and *Serratia spp*. [22], *Pantoea agglomerans* [23], [24], and *Chryseobacterium meningosepticum* [25]. Certain fungal species also infect mosquitoes *via* the cuticle [26], including *Metarhizium anisopliae* [27], *Aspergillus*, and *Streptomyces spp*. [28]. Other than that, viruses also exhibited potential as genetic control agents for malaria, such as mosquito *densonucleosis* viruses [29] and *Plasmodium vivax* Matryoshka RNA (Ribonucleic Acid) virus 1 [30]. Nevertheless, two bacterial genera, *Wolbachia* and *Asaia*, have garnered significant attention in the scientific literature for their recurrent evaluation as suitable hosts in paratransgenesis.

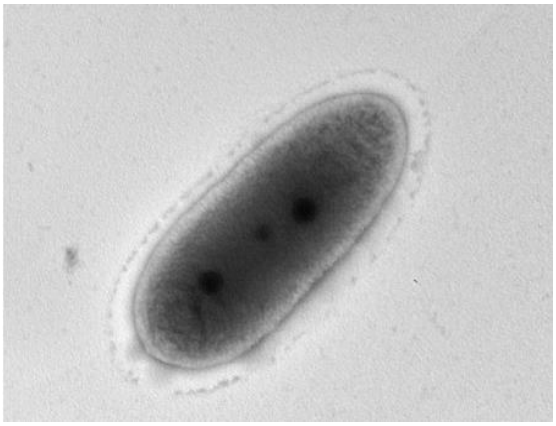

Figure 1. An *Asaia* sp. cell under electron microscopy. Adapted from [31].

Wolbachia and *Asaia* are frequently examined as prospective paratransgenic tools against malaria. While *Wolbachia* offers advantageous traits, it is unsuitable for the methodology employed in this project. Termed "reproductive parasites", these bacteria can manipulate the reproductive capabilities of their hosts to favor the bacteria, resulting in a decline in viable offspring [32]. Additionally, mosquitoes infected with *Wolbachia* experience a reduced lifespan [33]. While this diminished fitness could benefit population control, it limits the potential dissemination of the engineered toxin transgene. Moreover, the intracellular localization of *Wolbachia* contributes to their non-pathogenicity to humans, restricting their interaction with the primarily extracellular *Plasmodium* parasites [34]. This circumstance implies that the toxin transgene would likely be undesirably integrated into the mosquito cell. *Asaia*, classified as a gram-negative, rod-shaped bacterial genus, establishes colonization in the midgut, ovaries, and salivary glands of mosquitoes [35]. Notably, this bacterium has demonstrated both horizontal and vertical transmission within *Anopheles* mosquito populations and exhibits effectiveness across various *Anopheles* and *Aedes* species [36]-[39]. Human infections with *Asaia* are exceptionally rare, occurring predominantly in severely

immunocompromised individuals or instances of direct bloodstream injection [35]. A significant advantage of *Asaia* lies in its prevalence within the mosquito midgut and salivary glands, key organs targeted by the *Plasmodium* life cycle during the oocyst and sporozoite phases, respectively [34]. Unlike obligate endosymbionts like *Wolbachia, Asaia* thrives in extracellular environments [37], providing the proximity necessary for effective elimination of *Plasmodium* in their immediate vicinity. These advantageous characteristics positioned *Asaia* as the optimal candidate for hosting the paratransgenic strategy outlined in this project, establishing it as the most suitable symbiont with extensive research support in the literature. Previous studies often used *Asaia* with only constitutive promoter. Therefore, the engineered system would keep producing the antimalarial effectors. Antimalarial toxins such as scorpine, have antibacterial properties and therefore, can potentially kill the symbiont bacteria [38]. Thus, a system where the effector production is activated upon detection of *Plasmodium*, is needed. Moreover, the system also needs to prevent the death of the mosquitoes themselves, since the extinction of mosquitoes might have unknown environmental impact [39]. Therefore, this study tried to assess the feasibility in implementing the usage of Ti plasmid inside the *Asaia* bacteria as the symbiont for malaria paratransgenesis through protein structure analysis and binding. This process would provide and illustrate a new novel approach in tackling malaria, by providing protein binding parameters and analysis to possible protein targets that can be used to target *Plasmodium* through gene transfer. This study can be used in paratransgenesis endeavors, in order to address the problem of malaria prevention not just in Indonesia, but also in other parts of the world that are also impacted by malaria.

2. RESEARCH METHOD

This study has made use of protein database repositories as well as free-to-use *in silico* research tools accessible/available to be downloaded online. The protein databases for this project include websites such as: AlphaFold (https://alphafold.ebi.ac.uk/) and Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, http://rcsb.org). As for the research tools: PyMOL program (https://pymol.org/edu/), ClusPro website (https://cluspro.bu.edu/home.php), and PRODIGY (PROtein binDIng enerGY prediction) web server (https://wenmr.science.uu.nl/prodigy/). AlphaFold is a web database that not only hosts over 200 million 3D protein structures [40], but also computationally predicts the accuracy of the structures [41]. The model confidence analysis available on this server aid in filtering out inaccurate proteins from being included in this study.The PDB is an open-access, global archive that hosts experimentally validated, three-dimensional structures of biological macromolecules alongside the associated experimental data and metadata [42]. Within it holds a wealth of molecular structure data and structure confidence analysis invaluable to this project.

PyMOL software is a molecular graphic tool widely used to visualize proteins and other small molecules. It also possesses functions to edit molecules. This software serves twofold: its editing functions to extract only proteins of interest. In this study, PyMOL was used to assign chain labels before inputting the 3D structure into the PRODIGY server, which would be discussed in a later section. ClusPro is a widely used automated tool for protein-protein docking [43]. This web server reliably produces high-quality structures of protein-protein complexes that can compete with human predictor groups for a fraction of the time [44]. Cluspro is opted for its ease of use which does not require the user to have coding expertise. PRODIGY server is a web service that predicts the binding affinity of protein-protein complexes from their three-dimensional structures alone [45]. The UniProt ID of FecA is P13036. The corresponding IDs of the various *Plasmodium* proteins can be found in Table 1.

This study began with the preliminary data collection of 3D protein structures available online from AlphaFold and RCSB PDB. Two kinds of data were used: the 3D structure of FecA, obtained from the PDB databank, and a number of proteins of various human-pathogenic *Plasmodium* spp. from both AlphaFold and PDB. AlphaFold, however, only provided protein structure produced by using AI (artificial intelligence) with different confidence values, and therefore, only proteins with model confidences rated to be mostly “very high” were selected for the next step. RCSB-PDB, however,

provided protein structure results from X-ray crystallography. Both databases were used to complement each other, as not all proteins used for the analysis were available on both servers. Each *Plasmodium* protein undergoes molecular docking with FecA using the ClusPro program. The *Plasmodium* protein would bind to FecA, creating a 3D structure with 10 configurations (mod) that can be downloaded. From these models, the top 3 best models were chosen for the analysis that can be seen in Table 1. This docking result was then transferred to PyMOL to be assigned a chain label. This chain label allowed binding affinity analysis later with PRODIGY. This chain label needs to be assigned manually using PyMOL so PRODIGY can recognize the two proteins that are used for the binding affinity analysis. The labeling was done by entering "alter (protein name), chain="label name"" in the PyMOL command box after selecting the protein chain that needed to be labeled. The final step was to select the candidate *Plasmodium* compounds to serve as biomarkers. This selection involved a binding affinity analysis. The binding affinity analysis was done using the PRODIGY server with a unit of kcal/mol. The protein-protein complex that resulted from the ClusPro molecular docking can be submitted directly to the server, after being processed in PyMOL first. PRODIGY would analyze the complex and report the binding affinity result. The more negative the binding affinity is, the stronger the interaction between the ligand and its receptor. One would prefer a lower value for the best results. Cluspro repeats the analysis 10 times, ordering them based on the most negative results.

In order to know how significant the binding affinity is, the P-value was calculated with the help of a benchmark to determine whether the resulting binding affinity was strong or weak. The benchmark was obtained from another study that also utilized protein-protein docking analysis and had relatively strong binding affinity results [46]. With this analysis, it can be known how strong the resulting binding affinity is compared to other results. The P-value calculation was done in Microsoft Excel by using the statistical function provided by the software. The calculation utilized data from the COVID-19 *in silico* study as a benchmark for the binding affinity. According to research conducted by previous studies, different variants of SARS-CoV-2 have different binding affinity towards ACE-II (Angiotensin-converting enzyme 2) [47]. The Delta variant, Omicron BA.1 variant, and Omicron BA.2 variant each have -12.5 kcal/mol, -11.1 kcal/mol, and -12.6 kcal/mol binding affinity, respectively. This data was used for a one-tailed t-test to obtain the P-value between the COVID-19 dataset and the resulting binding affinity data from the FecA to the sample docking. The P-value was obtained by performing a one-tailed t-test inside Microsoft Excel with an alpha value of 0.05. Hypotheses used for the test include "Ha: binding affinity from another study < tested binding affinity results" and "Ho: binding affinity from another study > tested binding affinity results."

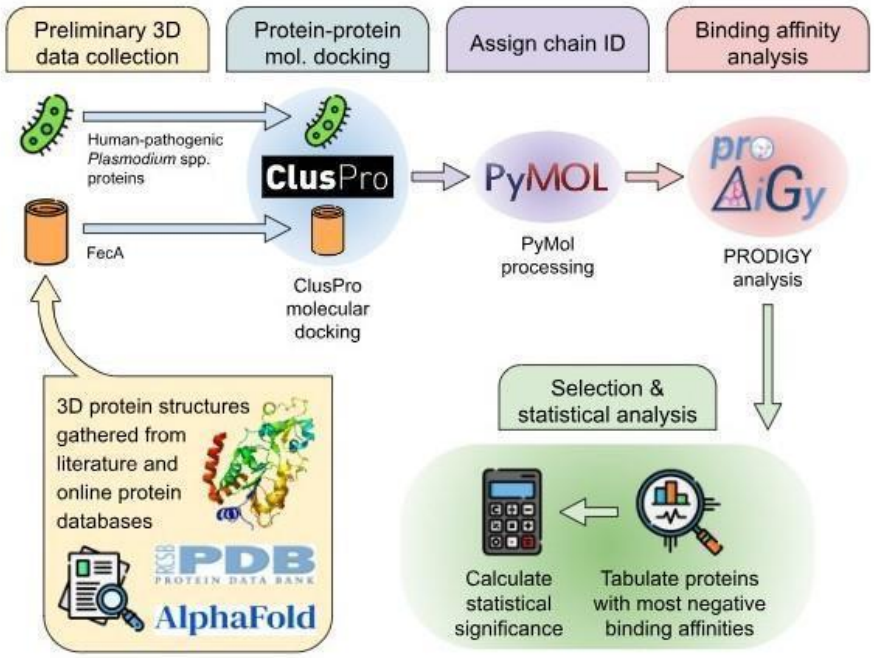

Figure 4. Research framework visualizing project methodology.

3. RESULTS AND DISCUSSIONS

Agrobacterium tumefaciens, known for causing crown-gall disease in plants, owes its pathogenic prowess to the presence of large plasmids, particularly the Ti (tumor-inducing) plasmid. This Ti plasmid boasts a repertoire of genes, with a crucial section known as the T-DNA (Transfer-DNA), responsible for instigating tumor growth in plants [48], [49]. The breakthrough came in 1983 when Hoekema et al. demonstrated the versatility of the Ti plasmid by replacing its oncogenic region with desired genes, effectively exploiting the 25 bp inverted repeats flanking the T-DNA for successful transfer to plants [50]. This mechanism, facilitating horizontal gene transfer across biological kingdoms, paved the way for *Agrobacterium*-mediated plant transformation.

In the *Agrobacterium*, acetosyringone plays a pivotal role, serving both as an attractant for the bacteria and as a trigger for the Ti plasmid's gene transfer mechanism. The signal molecule also kicks off a biochemical chain reaction that leads to the triggering of gene transfer mechanisms. Known as the virulence (vir) genes, the proteins they encode are integral in the transfer of the T-DNA region. One vir gene, known as chromosomal virulence gene E (ChvE) [51], is responsible for coding an important cell membrane protein called VirA [51]. This protein holds an essential function — it is the sensor that detects extracellular signal molecules. Sugars bind to VirA which induces the protein to phosphorylate a cytoplasmic protein called VirG [51], [52]. The phosphorylated VirG is now active. It acts as a transcriptional activator for other vir genes [53]. Upstream of every vir gene lies a 12 base pairs sequence located at its 5' end; this DNA region is known as the vir box [54]. VirG cooperatively binds to this vir box, helping RNA polymerase to transcribe the vir gene ahead of VirG gene [55].

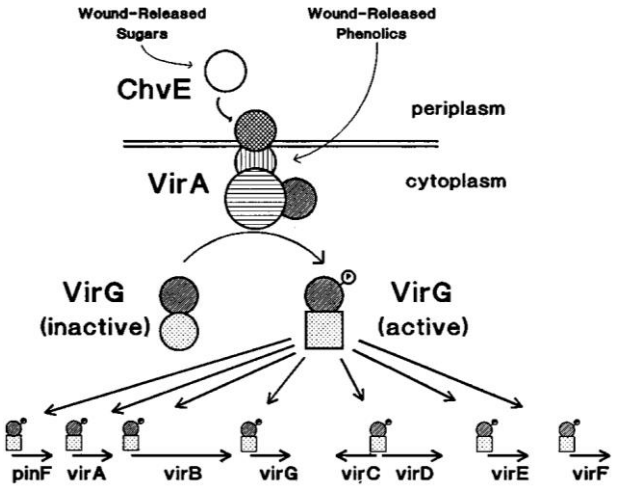

Figure 2. Plant wound molecules would bind to VirA, activating it. VirA then phosphorylates VirG. Once phosphorylated, VirG would induce the transcription of the rest of the vir proteins. Adapted from [56].

All together, the various vir proteins facilitate the gene transfer. First, T-DNA is replicated and the single-stranded (ss) copy of it excised. This ssT-DNA is then transferred into the plant cell in a mechanism similar to bacterial conjugation and smuggled into the nucleus (Figure 2). There, the tumour-inducing genes would randomly be incorporated into the plant chromosome and proceed to be expressed by the host cell. This mechanism has a potential to be used in malaria paratransgenesis for transferring genes that could kill *Plasmodium*.

Conventionally, in *Agrobacterium tumefaciens*, the role of biosensor is delegated to the VirA/VirG receptor mechanism [57]. This pair of proteins, unfortunately, is unsuitable for this project. In *Agrobacterium*, the typical function of VirA, which is located in the inner membrane, is to detect the presence of plant sugars. These sugars can diffuse through the outer membrane of the gram-negative *Agrobacterium* [51], [58]. VirA then transfers the signal to VirG in the cytoplasm [49], [57]; thereafter, the activated VirG acts as a transcriptional activator that binds to the vir box promoter to transcribe the critical vir proteins that facilitate gene transfer [56], [57]. However, the objective of this strategy is to detect the presence of *Plasmodium* proteins using a biosensor. These are far too large to bypass the outer membrane of *Asaia* and reach the VirA biosensor. A different biosensor mechanism would have to be utilized — one that is located in the outer membrane but is part of a protein pathway capable of transferring a signal through the inner membrane and into the cytoplasm; and likewise the pathway have to end in a protein that acts as a transcriptional activator like VirG. Fortunately, a kind of TonB-dependent iron transporter originating from *Escherichia coli* known as FecA (Fe(3+) dicitrate transport protein A) perfectly suits our needs. The VirA/VirG complex would be replaced by the FecA-FecR-FecI receptor complex. FecA is an outer membrane receptor that acts as a biosensor to detect *Plasmodium* biomarkers, replacing the role of VirA. FecA then transfers the signal to FecI at the inner membrane, then from FecI to FecR at the cytoplasm. FecR itself is also a transcriptional activator which binds to the FecABCDE operon promoter [58]. Thus, in our recombinant Ti plasmid, replacing the vir box, the vir genes that facilitate gene transfer would be instead headed by this FecABCDE promoter.

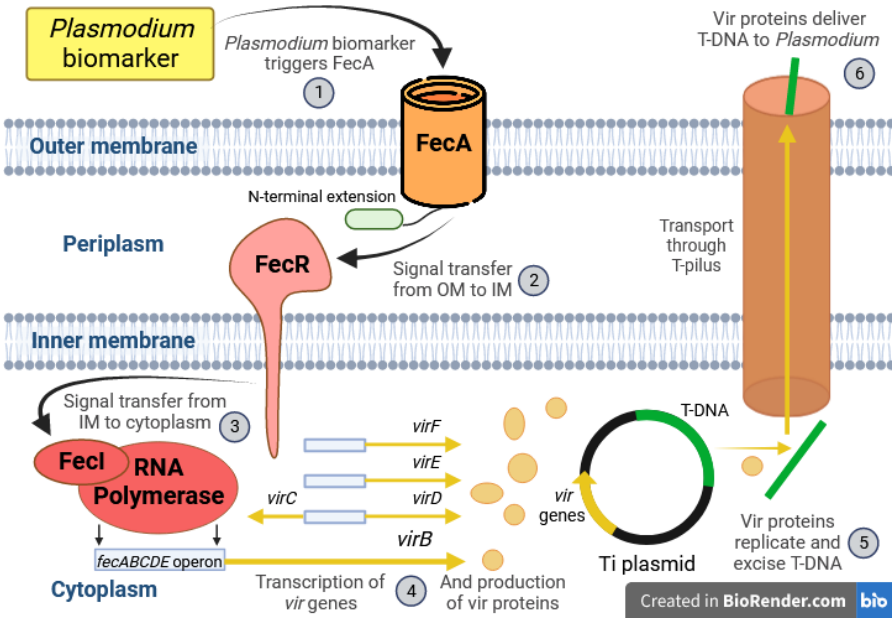

Figure 3. Proposed recombinant pathway. Adapted from [57], [58]. Created with BioRender.com (https://www.biorender.com/)

In order to assess the binding affinity of FecA, molecular docking was utilized in this study. Molecular docking is used to virtually predict the formation of a complex between a pair of binding partners. These partners typically include biological macromolecules like proteins, DNA (Deoxyribonucleic Acid), RNA, or peptides, as well as small compounds such as natural ligands and drugs. It finds frequent application in the field of structure-based drug design, enabling the anticipation of how ligands interact with a target protein. In the context of structure-based small-molecule docking, a small ligand is situated within the binding site of the target protein, and the resulting docking arrangement is evaluated using a dedicated scoring function. This function assigns a score to each arrangement, which is subsequently employed to rank different ligands and their corresponding positions. The docking process can be methodologically divided into two primary phases: pose generation and scoring. The former encompasses methods for generating diverse conformations of ligands and proteins while aligning these ligand structures within the protein's binding site. The latter, scoring, is crucial for quantitatively assessing the quality of the predicted positions. The score of this process is often referred to as binding affinity.

The binding affinity refers to the intensity of the interaction or strength of the connection between two or more reversibly binding molecules. This measurement is critical for determining what biological roles these proteins perform, such as signal transmission, cell control, and immune response [59]. In this study, binding affinity analysis allows for determining suitable ligands within the oocyte and sporozoite stage of *Plasmodium* that could bind to a specific region within FecA (Table 1). With a negative-valued binding affinity, it indicates a thermodynamically favorable ligand-receptor interaction that has a strong affinity binding to the protein, and a stable complex that can induce further signaling cascade [60].

This research has found that out of the initial 15 ligands found within *Plasmodium*; MAOP (Membrane attack ookinete protein), SOAP (Secreted ookinete adhesion protein), and CTRP (Circumsporozoite and TRAP-related protein) are not compatible with *FecA* through ClusPro protein-protein binding. This incompatibility may be attributed to the conformational formation of the 3 protein structures preventing any form of protein-protein binding towards FecA. This problem is not present in the other 12 proteins where protein-protein binding was observed. It was also observed that out of the 12 proteins; PPM5 (Metallo-dependent protein phosphatase) model 5 has the highest binding

affinity based on individual model, while GGCS has the highest binding affinity based on average affinity. This would mean GGCS (Gamma-glutamylcysteine synthetase) may have the highest general affinity towards FecA. However, other proteins may also have a high general affinity as well due to the selection of models used in this experiment based on the confidence level calculated in ClusPro, and the binding site of the protein. GGCS is a part of the glutathione redox system, one among many antioxidant systems responsible for detoxifying the *Plasmodium* cell from ROS (Reactive Oxygen Species), protecting the cell from external and internal oxidative stress [61]. Nevertheless, the binding of the 12 proteins has shown the presence of several ligands for FecA to bind to. Thus, it can be safely presumed that expression of *FecA* in *Asaia* would introduce a signaling cascade from the ligand within *Plasmodium* to *FecA*, resulting in the toxin plasmid transfer into the *Plasmodium*.

The P-value method was used to test the significant level of the binding affinity of the selected *Plasmodium* biomarker towards FecA in comparison to the COVID-19 spike protein affinity for ACE-II. The P-value lower than 0.05 indicates a significant result, or the Ho is rejected, while the P-value 0.05 indicates a non-significant result, which means the Ho is not rejected. ACE-II was chosen as the binding affinity reference in this research because this receptor serves as the gate for the SARS-CoV-2 virus to enter lung cells, and it was already widely studied during the COVID-19 pandemic as one of the disease that has a high infectious rate, which indicates that the virus and the receptor have a solid binding rate [59]. The results for the binding affinity analysis are shown in Table 1. Additionally, FecA and ACE-II have a somewhat similar mode of action, where activation of the proteins leads to the recruitment of protein for further signal transduction. In the case of FecA, receptor binding leads to the recruitment of the TonB protein in the TonB-box, which activates the TonB-dependent signaling cascade [62]. On the other hand, ACE-II receptor binding induces recruitment of the ADAM-17 (A disintegrin and metalloprotease 17) and TMPRSS2 (Transmembrane protease serine 2) for the ACE-II endodomain and C-terminal domain cleavage respectively [64]. The p-value analysis in this research resulted in 4 significantly different protein affinity out of the 12 protein bindings. When comparing the average binding affinity of the 4 proteins and the average affinity of ACE-II, the average binding affinity of the 4 proteins are found to be higher. Consequently, this shows the binding affinity of the 4 proteins are significantly higher than the binding affinity of ACE-II.

Table 1. Binding Affinity Results

No.	Protein Name	Binding Affinity (kcal/mol)	Average Affinity	P-Value in comparison with COVID-19 spike protein affinity to ACE-II (~0.05)	UniProt ID	Protein to FecA 3D Binding Structure
1	MAOP (Membrane attack ookinete protein)	Not compatible	-	-	Q5XLC0_PLABE	-
2	SOAP (Secreted ookinete adhesion protein)	Not compatible	-	-	A0A6M3BCB2	-
3	CTRP (Circumsporozoite and TRAP-related protein)	Not compatible	-	-	A0A6M3BCM1	-
4	CelTOS (Cell-traversal protein for ookinetes and sporozoites)	Mod 1 = -12.8 Mod 2 = -13.3 Mod 3 = -13.9	-13.33	0.058 (insignificant)	A0A6H0C7T1	(see Appendix 1-3)
5	P25	Mod 0 = -11.7 Mod 1 = -12.3 Mod 2 = -11.6	-11.87	0.365 (insignificant)	A0A1K9ZUQ0	(see Appendix 4-6)

No.	Protein Name	Binding Affinity (kcal/mol)	Average Affinity	P-Value in comparison with COVID-19 spike protein affinity to ACE-II (~0.05)	UniProt ID	Protein to FecA 3D Binding Structure
6	P28	Mod 1 = -11.5 Mod 4 = -16.1 Mod 5 = -16.5	-14.70	0.128 (insignificant)	Q8IJ96	(see Appendix 7-9)
7	SHLP1 (Shewanella-like protein phosphatase)	Mod 3 = -12.1 Mod 5 = -12.8 Mod 9 = -16.0	-13.63	0.156 (insignificant)	A0A1C6X2Z2	(see Appendix 10-12)
8	PPM5	Mod 3 = -19.6 Mod 5 = -23.3 Mod 10 = -17.0	-19.97	0.026 (significant)	A0A2I0BWX8	(see Appendix 13-15)
9	GGCS	Mod 0 = -23.2 Mod 3 = -20.1 Mod 5 = -17.8	-20.33	0.018 (significant)	Q8WP96	(see Appendix 16-18)
10	FabB/F (Beta-ketoacyl-[acyl-carrier-protein] synthase I)	Mod 0 = -16.9 Mod 1 = -13.3 Mod 4 = -14.0	-14.73	0.056 (insignificant)	Q965D4	(see Appendix 19-21)
11	CSP (Circumsporozoite protein)	Mod 0 = -9.9 Mod 1 = -16.0 Mod 2 = -12.5	-12.80	0.364 (insignificant)	P08677	(see Appendix 22-24)
12	IMC1a (Inner membrane complex protein)	Mod 0 = -20.1 Mod 1 = -14.5 Mod 2 = -16.5	-17.03	0.042 (significant)	Q5QT36	(see Appendix 25-27)
13	LAP1/CCp3/SR (LCCL/lectin adhesive-like protein 1)	Mod 0 = -16.5 Mod 1 = -14.3 Mod 3 = -20.4	-17.07	0.049 (significant)	Q8IM24	(see Appendix 28-30)
14	c-CAP (Cyclase-associated protein)	Mod 0 = -14.1 Mod 1 = -13.2 Mod 2 = -11.5	-12.93	0.200 (insignificant)	D3TTC3	(see Appendix 31-33)
15	GST (Glutathione S-transferase)	Mod 0 = -12.1 Mod 3 = -11.8 Mod 5 = -11.1	-11.67	0.263 (insignificant)	Q0ZS46	(see Appendix 34-36)

Furthermore, It is argued that although only 4 proteins are significant, the 8 other proteins can be a potential ligand for FecA as well. This argument comes from the fact that the binding affinity between FecA-ligand and ACE-II are similar, as can be seen from the p-value being higher than 0.05. Moreover, based on the previous study, binding affinity of less than -10 kcal/mol is already considered as strong binding [65]. Hence it is believed the 12 proteins which can bind to FecA may also be a potential binding target for the *Plasmodium* ligand recognition system. One concern that may arise would be whether the significant binding affinity would result in the inability of FecA and the protein to separate. It is postulated that the inability of separation would not affect the working of the ligand recognition and/or signaling cascade system, as a constant binding would result in the constant transfer of toxin-encoded plasmid into the *Plasmodium*. Therefore, the signaling cascade would continue until the *Plasmodium* loses its biological function; in which case the toxin-encoded plasmid expression within the *Plasmodium* would stop, indicating the end of the signaling pathway.

Finally, a plasmid design incorporating all the concepts discussed in this research, including *Plasmodium* detection, infection, and elimination was shown (Figure 5). The detection system utilizes a constitutive promoter, labeled pro1, which expresses FecA, FecR, and FecI. Signal detection is done by FecA, and is transduced from FecA to FecR and into FecI. The signal from FecI regulates the

activation of the infection system's promoter, labeled pro2, which expresses the Vir operon. The operon facilitates the T-DNA region (labeled with the green stick) cleavage and transfer to *Plasmodium*. The transferred T-DNA region contains a promoter specific to *Plasmodium*'s RNA polymerase, labeled pro3, which expresses scorpine gene. Expression of the gene results in scorpion toxin, which eliminates *Plasmodium* with every gene expression. Paratransgenesis tends to also have the potential to kill the host vector along with bacteria vector used for the paratransgenesis. Previous study did not include a biosensor system and only engineered symbionts that produce the toxin molecule constitutively, potentially killing the mosquitoes and the bacteria vector [66], [35]. In this study, a new approach by using Ti plasmid might hold the answer to this problem. This novel approach therefore, can provide a potential solution to eliminate malaria without killing the *Anopheles* mosquitoes. Until today, there is still no convincing evidence that killing all mosquitoes would have no environmental impact, nor any study that could efficiently and effectively target *Anopheles* specifically to eliminate malaria [39]. Therefore, adding a biosensor system to only target the *Plasmodium* parasites or at least mosquito individuals that carry the parasites, can provide at least an interim solution before any decision can be made to eliminate *Anopheles* specifically.

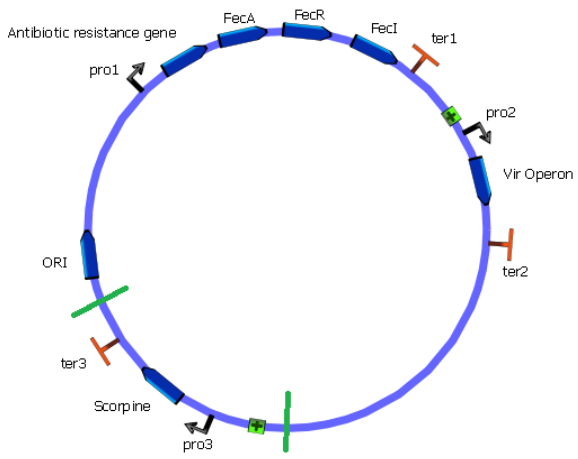

Figure 5. Design concept for the engineered Ti plasmid

However, one potential concern regarding the system involves guaranteeing the effective delivery and long-term presence of the bioengineered *Asaia* bacteria within the mosquito population. Achieving this step could prove complex due to various factors, including the bacteria's ability to persist and successfully establish colonies in the mosquito gut. As the parasite *Plasmodium* progresses within its invertebrate hosts (vectors), its quantity typically decreases, starting from 103–104 gametocytes and diminishing to 102–103 motile ookinetes, eventually reaching less than 5 oocysts [67]. Targeting and intervening at this bottleneck stage could be crucial in preventing parasite transmission. Thus the sessile nature of the *Plasmodium* oocyst and its extended incubation period makes it a desirable target [68]. Another possible concern is the lack of knowledge regarding the effects of the binding. This includes whether the binding would result in the activation of the signaling cascade, or inhibition of the protein's function. Therefore, future research may try to assess the result of protein binding through proper wet-laboratory based protein binding.

4. CONCLUSION

This study is dedicated to identifying *Plasmodium* proteins with strong binding affinity to a wide range of targets, initiating gene transfer within the Ti plasmid. Additionally, the study also proposes a plasmid design featuring a modified FecA gene carrying a toxin transgene. Among the initial 15 ligands, 12 demonstrated protein-protein binding, with PPM5 mod 5 exhibiting the highest individual affinity

and GGCS demonstrating the highest average affinity. While four proteins showed significantly different affinities, the remaining eight, although not statistically significant, still hold promise as potential FecA ligands. The resemblance in binding affinity with ACE-II suggests that all 12 proteins could serve as viable targets for the *Plasmodium* ligand recognition system, broadening the scope of the investigation. The suggested plasmid and recombinant pathway design integrates key elements discussed in the research, covering *Plasmodium* detection, infection, and elimination. The detection system utilizes a constitutive promoter expressing FecA, FecR, and FecI. The signal from FecI governs the activation of the infection system's promoter, expressing the Vir operon that facilitates T-DNA region cleavage and transfer to *Plasmodium*. The transferred T-DNA region, housing a *Plasmodium*-specific RNA polymerase promoter, expresses the scorpine gene. Consequently, scorpine gene expression leads to the production of a scorpion toxin, efficiently eradicating *Plasmodium* with each gene expression occurrence. In short, using Ti plasmid in *Asaia* for malaria paratransgenesis might potentially be feasible since the proposed biosensor system can bind to the analyzed ligands. However, the true feasibility of our approach warrants further validation, particularly in an in vitro environment, to assess its efficacy and address potential limitations. Future research could focus on refining the biosensor system, exploring its in vitro efficacy, and investigating challenges associated with in vivo implementation. Through these efforts, we aim to contribute to the development of effective strategies for malaria control and eradication.

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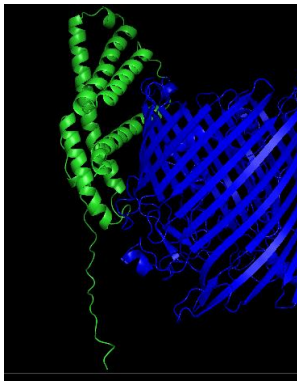
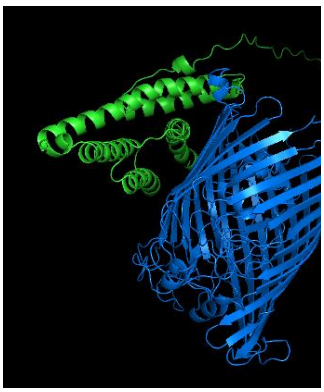
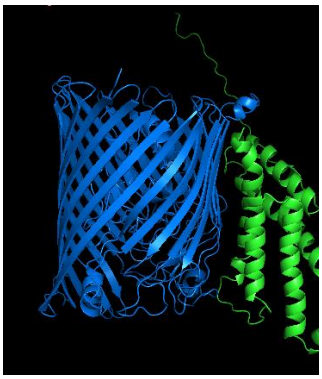
APPENDIX



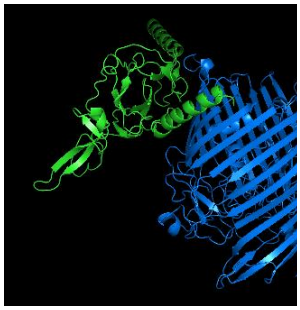
Appendix 1. CelTOS-FecA Model 1



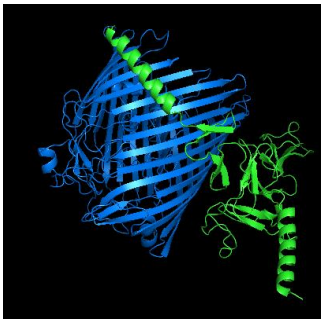
Appendix 2. CelTOS-FecA Model 2



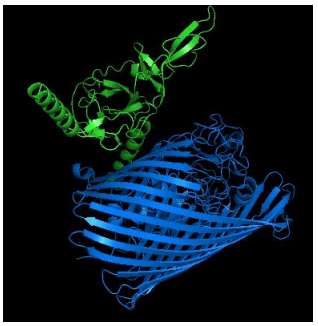
Appendix 3. CelTOS-FecA Model 3



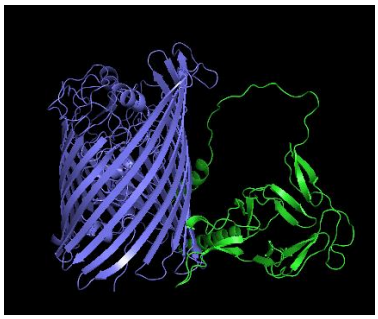
Appendix 4. P25-FecA Model 0



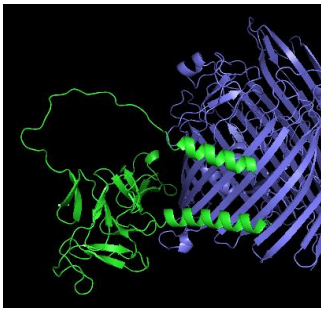
Appendix 5. P25-FecA Model 1



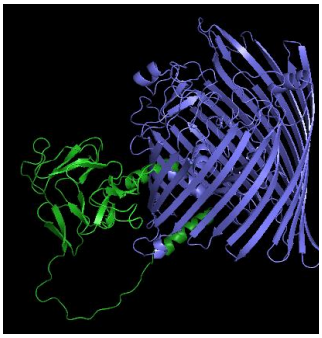
Appendix 6. P25-FecA Model 2



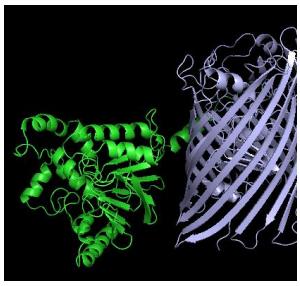
Appendix 7. P28-FecA Model 1



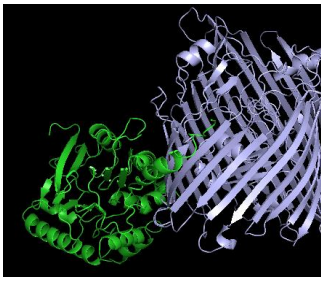
Appendix 8. P28-FecA Model 4



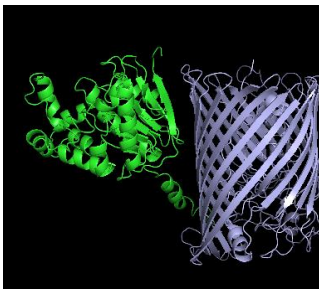
Appendix 9. P28-FecA Model 5



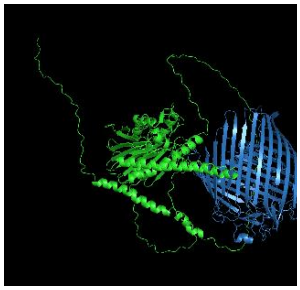
Appendix 10. SHLP1-FecA Model 3



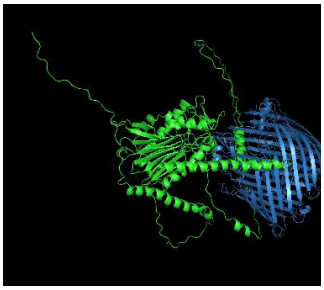
Appendix 11. SHLP1-FecA Model 5



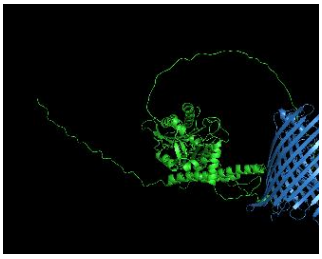
Appendix 12. SHLP1-FecA Model 9



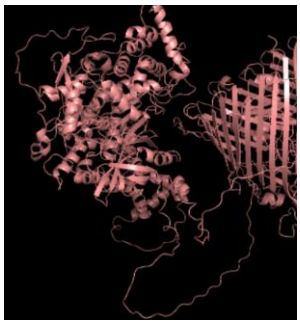
Appendix 13. PPM5-FecA Model 3



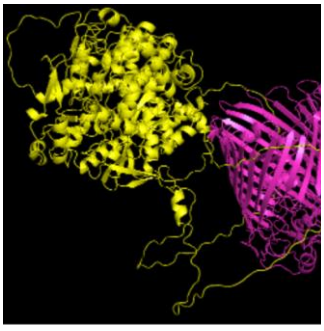
Appendix 14. PPM5-FecA Model 5



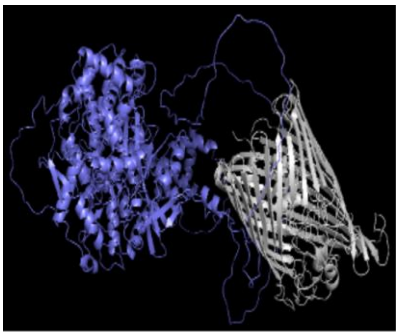
Appendix 15. PPM5-FecA Model 10



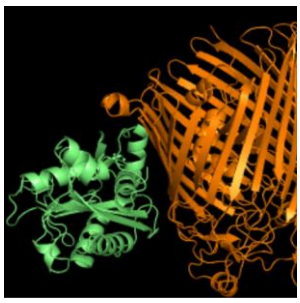
Appendix 16. GGCS-FecA Model 0

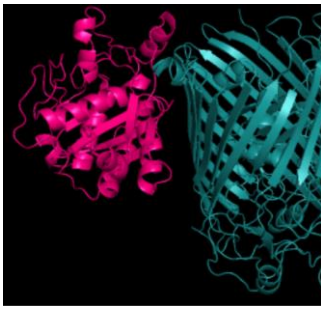


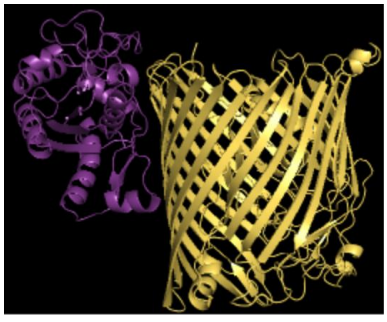
Appendix 17. GGCS-FecA Model 3



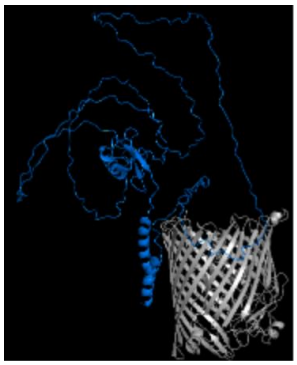
Appendix 18. GGCS-FecA Model 5



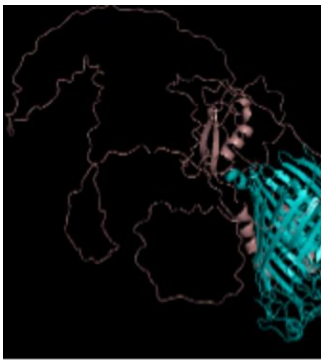




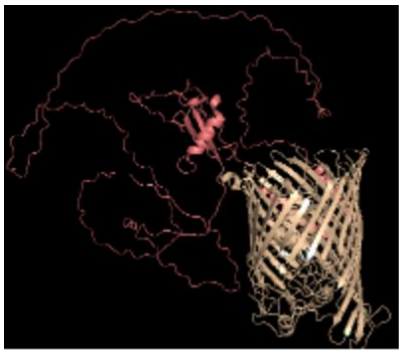
Appendix 19. FabB/F-FecA Model 0



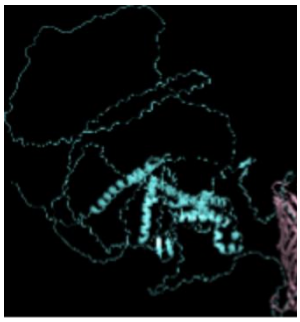
Appendix 20. FabB/F-FecA Model 1



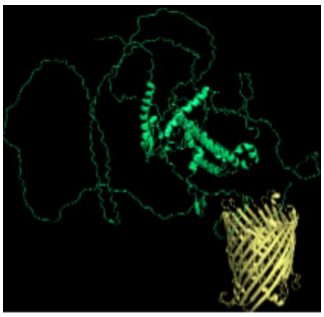
Appendix 21. FabB/F-FecA Model 4



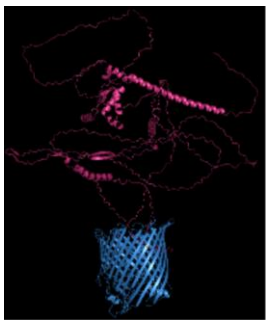
Appendix 22. CSP-FecA Model 0



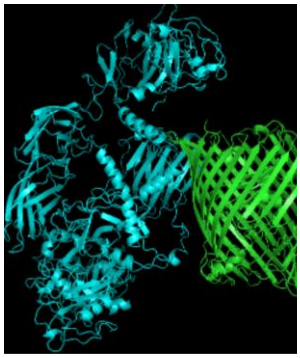
Appendix 23. CSP-FecA Model 1



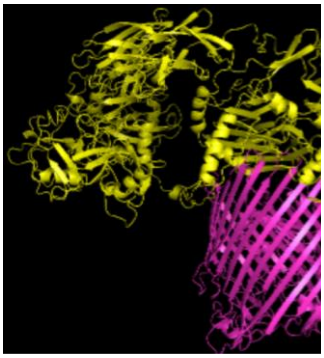
Appendix 24. CSP-FecA Model 2



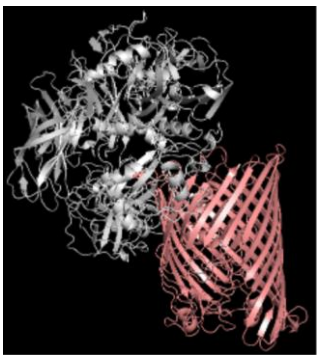
Appendix 25. IMC1a-FecA Model 0



Appendix 26. IMC1a-FecA Model 1



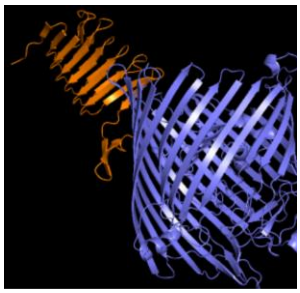
Appendix 27. IMC1a-FecA Model 2



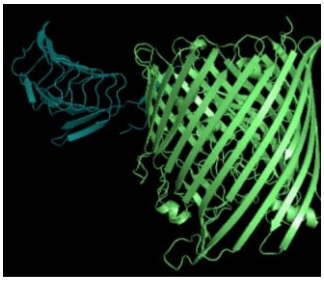
Appendix 28. LAP1/CCp3/SR-FecA Model 0

Appendix 29. LAP1/CCp3/SR-FecA Model 1

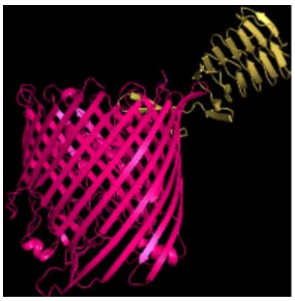
Appendix 30. LAP1/CCp3/SR-FecA Model 3



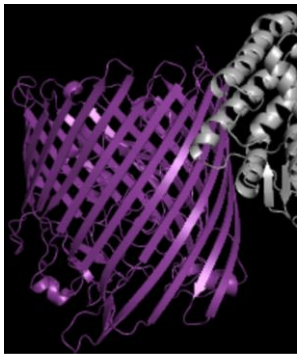
Appendix 31. c-CAP-FecA Model 0



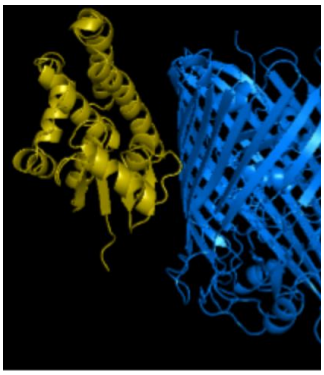
Appendix 32. c-CAP-FecA Model 1



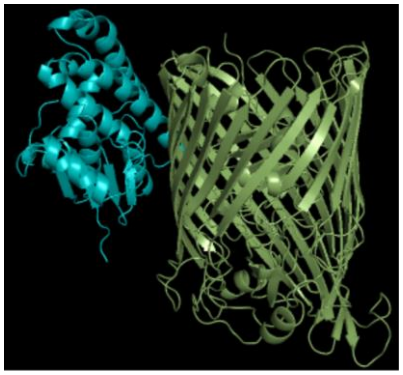
Appendix 33. c-CAP-FecA Model 2



Appendix 34. GST-FecA Model 0



Appendix 35. GST-FecA Model 3



Appendix 36. GST-FecA Model 5