Designing T-cell epitope-based vaccine against Eimeria infection in chicken using immunoinformatics approach

by

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Preface

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa, under supervision of Dr MA Adeleke and co-supervised by Dr M Okpeku. The research was financially supported by the National Research Foundation (NRF) of South Africa for financial support (Thuthuka Grant number: 112768 and S&F-Innovation masters scholarship Grant number: 123320).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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ABSTRACT

Chicken coccidiosis is the most significant ubiquitous, intestinal parasitic disease known to infect chickens globally. It is recognised for incurring significant production loss to the poultry industry, caused by single or multiple *Eimeria* spp. infections which threaten chicken welfare and productivity. The emergence of drug resistance in parasites and pathogenicity reversion has put pressure on the poultry industry to reduce chemoprophylactic drugs and live vaccines as preventive measures against coccidiosis. Recombinant DNA vaccines have shown promising results as an alternative option, but complete protection has not been reported highlighting the need for the design of new vaccine against this disease. In this study, *Eimeria* antigens Immune Mapped Protein-1(IMP1) and Microneme Protein-2(MIC2) were explored using reverse vaccinology and immunoinformatics tool to predict and design potential multiepitope vaccine candidate against coccidiosis. A total of 28 and 19 antigenic T-cell epitopes were predicted and used to construct two multiepitope vaccines with 610 and 512 amino acids for IMP1 and MIC2, respectively. The produced vaccines exhibited favoured characteristics for an ideal vaccine candidate; they were antigenic (Vaxijen score of 0.5989 and 0.5103), immunogenic (scores: 10.15 and 9 419), thermostable (instability index <40), and non-allergic. The presence of IFN-gamma and IL-4 inducing epitopes in the constructed vaccine enables vaccine to trigger a cellular and humoral response within the host. Molecular docking of designed vaccines with toll-like receptors (TLR4 and TLR5) to determine vaccine interaction and stability was confirmed by molecular dynamics simulation root-mean square deviation (RMDS) and root-mean-square fluctuation (RMSF) analysis. The designed vaccines induced immune response through production of cytokines and antibodies associated with tertiary response. When exposed to online immune simulation C-ImmSim, both vaccines produced potent immune response through production of IgG, Tc and Th cell and memory B-cells. The constructed multiepitope vaccine in this present study is highly promising and as such further experimental work should be done to confirm its suitability against chicken coccidiosis.

Keywords: Coccidiosis; *Eimeria*; Vaccine; Immunoinformatics; IMP1; MIC2

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CHAPTER ONE

1.1. INTRODUCTION

Coccidiosis is a parasitic intestinal disease globally recognised as a major contributor to significant economic loss in the chicken industry (Blake et al., 2020b; Geng et al., 2021). The disease is one of the most problematic and popular disease known to affect animal production, drastically reducing profitability while incurring a significant economic loss from prevention and treatments (Snyder et al., 2021). It is caused by *Eimeria* parasites in chickens, resulting in enteritis, bloody diarrhoea, malabsorption, and in severe cases may lead to high mortality (Blake et al., 2015). The economic loss resulting from the disease's impact globally was projected to exceed USD 3 billion over past years; however, recent literature has revealed a significant increase in global cost incurred by the poultry industry estimated at USD 13.6 billion/~£10.4 billion since 2016 (Blake et al., 2020a; Blake and Tomley, 2014; Dalloul and Lillehoj, 2006). The severity of coccidiosis correlates with the rapid epithelial invasion and reproduction of multiple *Eimeria* species within the chicken gastrointestinal tract. This impacts chicken gut integrity and disturbs the balance of the intestinal microflora (MacDonald et al., 2017). The invasion leads to impaired welfare and productivity, resulting in increased susceptibility to secondary infections due to exposure to other opportunistic pathogens (Ritzi et al., 2016).

The control of *Eimeria* in the commercial poultry industry has heavily relied on chemoprophylaxis and live or attenuated *Eimeria* formulations as a form of vaccination (Soutter et al., 2020). However, the continuous and prolonged mass administration of anticoccidial drugs in poultry to control the disease was reported to cause endogenous mutations in coccidian parasites, contributing to the emergence of parasite drug resistance and high cost for treatment and prevention measures (Tan et al., 2017). The observed constraints have led to a shift in the body of research to explore alternative strategies to combat coccidiosis, including the exploration of antigens such as Immune mapped protein-1 (IMP-1) and microneme protein-2 (MIC2) as suited candidates for recombinant epitope-based DNA vaccines.

To avoid chemoprophylaxis or antibiotics, vaccination of chickens is the most implemented alternative option in controlling coccidiosis (Blake et al., 2020a). Recombinant DNA vaccines containing parasite antigens have been used to induce a strong immune response, providing efficient defence against *Eimeria* challenge (Blake et al., 2015; Zhang et al., 2012). Research

shows that proteins involved in late development stages of *Eimeria* (e.g., merozoites and gametocytes) induce cellular and humoral immune responses contributing to chicken's natural immunity (Jenkins, 1998). Even though the effectiveness of these antigens was reported, different species of *Eimeria* elicit different immune responses, posing a challenge in coccidiosis as this prevents cross-species immunity. This triggers the need for the development of a vaccine that can stimulate protection against multiple species of *Eimeria*.

Antigen IMP-1 is characterized as a highly conserved membrane protein, and a protective antigen discovered in *E. maxima* (Blake et al.,2011; Jenkin et al., 2015; Tang et al., 2018). It was identified as an immunogenic and surface-expressed antigen responsible for conferring protection against apicomplexan parasites such as *Eimeria* maxima infections (Yin et al., 2013). Similar protective effects against *Eimeria* challenge have been reported about MIC2, a highly immunogenic and conserved protein antigen, expressed in early developmental stages (in sporozoite and merozoite), crucial for parasite invasion and replication (Blake et al., 2017; Yan et al., 2018). Rigorous research has been done on these antigens as promising alternative prevention method against *Eimeria*, with promising outcomes reported on stimulation of strong immune responses and protection of chickens (Kundu et al., 2017; Pastor-Fernández et al., 2020; Zhang et al., 2020). Application of immunoinformatic tools for the prediction of T-cell epitopes and vaccine design proved to save time, costs, and improved chances of designing vaccines. Recent trends show limited appropriate standard protocol for the development of vaccines against *Eimeria* in poultry that would lower development and production costs of vaccines for effective control of coccidiosis. Hence, this study explores the two *Eimeria* antigens using the immunoinformatic approach to design a potentially effective epitope-based vaccine.

1.2. PROBLEM STATEMENT

Chicken coccidiosis is an intestinal protozoan disease of economic importance, which results in serious health impact, reduced viability and global economic loss to chicken industry (Blake et al., 2020b). This disease is associated with reduction in feed intake, poor nutrient absorption by animals and high morbidity rate in severe cases where complications are often presented by secondary bacterial infections. Due to severe effects of coccidiosis experienced in poultry, there have been strategies deployed to control the disease like the use of prophylactic chemotherapy (anticoccidial drugs) and commercial vaccines such as live virulent, attenuated or live-tolerant vaccines. Vaccination of poultry has thus far remained the most effective and

commonly used control option for coccidiosis. With studies affirming the efficiency of these strategies, several throwbacks have been reported on these strategies such as emergence of drug resistant *Eimeria* parasites, high cost related to treatments, large scale production of live vaccines, as they are dependent on oocyst recovery from chickens and faeces (Zhou et al., 2013), restocking and partial immune protection of host from attenuated vaccines (Wondimu et al., 2019). The search for alternative measures has led to exploration of natural products and recombinant vaccines through immunoinformatic approach as novel options for control against disease. To develop novel vaccines, more attention should focus on understanding interaction of host and parasites, and host immunity in response to *Eimeria* infection (Ma et al., 2019). There is still paucity of crucial information on the interaction within host of antibody and T-cell response of pathogens towards currently available vaccines. These parasites consist of complex, rapid life cycles and large genomes which makes the development of vaccines that confer complete protection an extremely difficult task. This information is crucial in the development of long lasting and cost-efficient vaccines to control of coccidiosis.

1.3. OBJECTIVE

To predict and design T- cell epitope-based vaccine against *Eimeria* using immunoinformatic tools.

1.4. SPECIFIC OBJECTIVES

- To review the interaction of Eimeria infection and gut microbiota towards the control of chicken coccidiosis
- To screen for antigenic, immunogenic, conserved and cytokine-inducing T-cell ($CD8^+$ and $CD4^+$) epitopes in IMP1.
- To screen for antigenic, conserved and cytokine-inducing T-cell ($CD8^+$ and $CD4^+$) epitopes in MIC2.
- To construct multiepitope with linkers and adjuvants for both IMP1 and MIC2.
- Validation of efficacy of designed multi-epitopes as vaccines.

1.5. THESIS OUTLINES

This thesis is in five chapters and is prepared in the form of submitted articles and manuscripts in preparation as shown below.

- Chapter two (Article 1): Understanding the interaction between *Eimeria* infection and gut microbiota towards the control of chicken coccidiosis. This article is under review

in *Parasite*. The manuscript reviewed the interaction between *Eimeria* infection and gut microbiota towards chicken coccidiosis control

- Chapter three (Article 2): Designing T-cell epitope-based vaccine against *Eimeria* from Immune mapped protein-1 antigen using immunoinformatic approach. This manuscript is in preparation to be submitted to Computational Biology and Chemistry.
- Chapter four (Article 3): Designing T-cell epitope-based vaccine against *Eimeria* from Microneme protein-2 antigen using immunoinformatic. This manuscript is in preparation to be submitted to Infection, Genetics and Evolution.
- Chapter five: General Discussion, Conclusion and Recommendation

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CHAPTER TWO

Understanding the interaction between *Eimeria* infection and gut microbiota towards the control of chicken coccidiosis

2.1. Abstract

Coccidiosis is the most significant ubiquitous disease that infect chickens globally. This disease is caused by obligate apicomplexan parasites of the genus *Eimeria*. *Eimeria* invasion causes damage to intestinal epithelial cells and disrupts the gastrointestinal tract (GIT) environment, compromising the host's immune system and chicken welfare; consequently, incurring the greatest economic loss to the poultry industry. Disruption of the gut environment causes an imbalance in the gut microbial community leading to dysbiosis. This affects gut microbiota's composition and diversity, reducing the abundance of beneficial bacteria (Firmicutes and Lactobacillus) and driving increment of pathogens such as *Clostridium* species. The gastrointestinal tract consists of diverse and significant microbes that aid in nutrition and proper development of chickens. Any fluctuation observed in GIT compromises chicken health, making it vulnerable to diseases such as coccidiosis. To control coccidiosis, observation of gut microbiota interaction and exploration of natural additives such as probiotics and phytochemicals has been encouraged in the poultry industry to exhibit their beneficial effects concurrently with gut microbiota, without the presence of drug resistance. This review aims to provide insight into the interaction between *Eimeria* infection and gut microbiota towards chicken coccidiosis control. It also reviews the interaction of natural alternatives with gut microbiome in the control of *Eimeria*.

Keywords: Coccidiosis; *Eimeria*; chickens; gut microbiota; probiotics; phytochemicals.

2.2. Introduction

Coccidiosis is the most significant ubiquitous disease that affects the chicken industry globally caused by obligate intracellular parasitic protozoa belonging to the genus *Eimeria*. It is currently posing great economic loss exceeding 3 USD billions yearly due to; loss of productivity, high mortality and high treatment costs to control the disease (Stanley et al., 2014; Yang et al., 2019b). The disease is characterized by the colonization and overcrowding of intestinal mucosa of chickens by *Eimeria* spp, compromising chicken welfare and productivity. Infections caused by these parasites can be easily transmitted between hosts by direct faecal-oral route through ingestion of sporulated oocysts from contaminated feeds or litter (Hauck, 2017; Leung, 2018). Seven recognised *Eimeria* species cause coccidiosis at varying degrees of severity in chickens (Quiroz -Castañeda and Dantán-González, 2015). These species include *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella* being the most prevalent (Song et al., 2015). Each species has its host-specific spectrum, targeting specific sites in the gut and posing severe damage in host's absorption capability and growth (Kers et al., 2018). Turk (1982) reported on colonisation sites preferred by *Eimeria* spp., with upper small intestine (jejunum and duodenum) mainly infected by *E. acervulina, E. necatrix, E. maxima* and *E. praecox*. *Eimeria tenella, E. mitis* and *E. brunetti* infections occur in the caeca, ileum and colon, where the reaction of intestinal lymphoid tissue and intestinal damage by *E. tenella* results in significant blood loss at the acute stages of infection observed as haemorrhagic patches, increase in caecal protein and DNA which affects chicken weight gain and reduction in muscle mass (Fetterer et al., 2001; Leung et al., 2018).

Eimeria infection destroys host mucosal cells resulting in elevated cell permeability, nutrient and plasma protein leakage, impaired digestion and protein absorption, contributing to clinical and subclinical effects of coccidiosis (Nabian et al., 2018; Vieira et al., 2020; Yang et al., 2020). This compromises chicken welfare as it disrupts the host's gut homeostasis; causing significant malabsorption, reduced feed conversion and weight gain, and overall productivity in chickens (Leung et al., 2018; Quiroz -Castañeda and Dantán-González, 2015). The severe intestinal damage caused by *Eimeria* colonisation not only affects epithelial cells, but it causes great disruption of gut microbial communities in the gastrointestinal tract (GIT), promoting invasion and spread of other opportunistic microorganisms such as *Clostridium perfringen*, causing susceptibility of infected chickens to secondary diseases, increasing chicken mortality (Antonissen et al., 2016; Hauck, 2017; Macdonald et al., 2019). It was reported that *Eimeria* invasion disrupts the gastrointestinal tract (GIT) environment, resulting in an imbalance in the

gut microbial community known as dysbiosis (Ducatelle et al., 2015). The gastrointestinal tract consists of diverse and significant microbes that aid in nutrition and proper development of chicken. Fluctuation and imbalance of microbial communities already existing in chicken's GIT impact the host's health and vulnerability to diseases. The control of these diseases requires high costs from treatment strategies and effective control. This has led to the exploration of various coccidial treatment strategies over the years, such as implementing chemoprophylaxis and live-attenuated vaccines. However, these strategies have proven to be effective with significant drawbacks, including drug resistance and possible parasite reversion to its virulent state. This review aims to understand the interaction between *Eimeria* infection and gut microbiota towards chicken coccidiosis control. Alternative strategies that are safe, antibiotic-free and cost-effective to control coccidiosis in poultry are also reviewed.

2.3. The function of gastrointestinal gut microbiota

Gastrointestinal tract (GIT) of chicken serves a critical role in digestion and protection. It is crucial in converting ingested feeds into nutrients essential for maintenance, growth, and reproduction (Baldwin et al., 2018). It also aids in developing immune response needed to prevent intestinal colonization by pathogenic or opportunistic microorganisms through pathogen exclusion (Haung et al., 2018; Stanley et al., 2014; Tsukahara et al., 2018; Wu et al., 2019). GIT comprises a complex and diverse microbiota in chickens, including bacteria, viruses, archaea, and fungi. Bacteria are predominant in GIT and have a beneficial symbiotic interaction with the host, crucial for the chicken nutrition, health and production (Shang et al., 2016). These microorganisms attach to the epithelial wall and trigger the immune system's maturation consisting of the mucus layer, epithelial monolayer, and other immune cells. The layers form a protective barrier to combat opportunistic bacteria colonisation (Baldwin et al., 2018). They also produce vitamins (e.g., vitamin K), short-chain fatty acids (acetic acid), organic acids (e.g., lactic acid) and other complex compounds that provide nutrients and energy essential for nutrition and protection of the animal (Stanley et al., 2014).

Microbes located in the GIT mainly maintain homeostasis of the intestinal mucosa by digestion of food sources, providing the energy needed to induce the intestinal immune system to fight against aggressions of other microorganisms (Chen et al., 2020; Wilkinson et al., 2016). A standard or balanced gut microbiota reduces host susceptibility to pathogenic parasites like *Eimeria* spp. (Huang et al., 2018). Commensal bacteria regulate nutrient absorption and protect the host by preventing pathogen colonization through competitive exclusion (Yan et al., 2017).

They also regulate immune activity by controlling mediators secreted by the mucosa membrane, triggering helper cells (Pourabedin and Zhao, 2015; Tsukahara et al., 2018). A fully developed immune system secretes proteins such as immunoglobin A (IgA) essential for regulating bacterial composition in the gut (Shang et al., 2016).

Microbiota located in the caeca or colon (distal gut) such as Ruminococcaceae, produces energy and nutrients through the degradation of substrates such as non-starch polysaccharides to simple sugars using hydrolytic enzymes (Borda-Molina et al., 2018). *Faecalibacterium* aids in the fermentation of these sugars results in the production of substances like short-chain fatty acids (SCFAs), i.e., butyrate and essential amino acids available for host consumption (Wu et al., 2019). Production of butyrate in chickens is crucial for reducing chronic inflammation and relieving *E. tenella* infection (Chen et al., 2020). The presence of SCFAs not only serves as the energy and carbon source for broilers, but it also aids in the regulation of blood flow, which stimulates cell growth in the intestinal lining (Clavijo and Florez, 2017). The degradation or fermentation of carbohydrates and polysaccharides releases easily accessible energy to other microorganisms, allowing different metabolic processes to occur (Pourabedin et al., 2015).

2.4. Normal gut anatomy and microbiota

For chickens to effectively utilize end-products resulting from metabolic processes, a balanced microbial community is crucial to facilitate internal interaction between host and diet (Wilkinson et al., 2016). Chicken's GIT is divided into sections consisting of diverse and unique microflora playing different metabolic functions; these sections include crop, proventriculus, gizzard, small (jejunum and duodenum) and large intestines (ileum, caeca and colon) (Pourabedin and Zhao, 2015; Rubio, 2019; Turk, 1982). These sections harbour diverse communities of commensal, symbiotic and pathogenic microorganisms, e.g., crop, duodenum, and gizzard share almost similar microbiota composition (about 99% Lactobacilli population) (Gharib- Naseri et al., 2019). The bacterial composition and diversity within the GIT influence intestinal functions such as digestion and nutrient absorption. A balanced chicken GIT is predominated by *Firmicutes, Tenericutes, Bacteroidetes,* and *Proteobacteria* (Borda-Molina). These commensal bacteria are located in the crop, with *Lactobacillus* exhibiting the highest diversity. They aid in the hydrolysis of starch and fermentation of lactate (Bortoluzzi et al., 2019). They are also present in high abundance in the ileum, facilitating nutrient absorption (Rodriguez-Sanchez et al., 2019). It was observed that even though the composition of gut microbiota located in the ileum is more distinct and dominated by *Lactobacillus, C.*

Arthromitus, *Enterococcus*, and *Clostridium*, it is also less stable compared to other sections of the GIT (duodenum and the jejunum) (Mohd Shaufi et al., 2015; Pourabedin et al., 2015).

The cecum is known to have the most remarkable taxonomic diversity and shelters more diverse microflora, including anaerobes, e.g., *Clostridium, Bacteroides, Proteobacteria, Actinobacteria* and *Firmicutes* (Rubio, 2019; Stanley et al., 2014; Zhou et al., 2020). As broilers advance in age, caeca gut microbiota undergoes significant changes resulting in a richer and stable microbial community (Gong et al., 2019). These microbes play a crucial role in the digestion of essential complex compounds such as cellulose, starch and other polysaccharides (Vieira et al., 2020). Microbial richness and diversity can be observed in the cecum during the second week, where most microbes from phylum Proteobacteria are replaced and dominated by *Firmicutes -Lachnospiraceae* and *Ruminococcaceae* (Kubasova et al., 2019). Previous research shows that changes in caecal microbial communities are likely due to microbes transferred through direct contact with the adult hen to offspring and chick ingesting the mother's faecal material (Turk, 1982). During the direct transfer, hens are significant donors of *Bacteroidetes* and *Actinobacteria* (Kubasova et al., 2019). Development and changes in gut microbiota often compromise the immune system and health of chicks, leaving them susceptible to various bacterial and parasitic infections, e.g., *Salmonella, C. perfringens,* and *Eimeria*. Direct contact of offspring with old hens plays a vital role in microbial development and maturation regardless of environmental factors (feed and litter) (Pourabedin and Zhao, 2015). Since microbiota becomes more diverse and stable as birds grow, changes observed in the gut, whether genetically transmitted by breeder birds or influenced by the environment, often result in a build-up of harmful bacteria which disrupts gut microbiota over beneficial ones (Antonissen et al., 2018; Tilocca et al., 2016).

2.5. Effect of coccidian (*Eimeria*) infection and pathogenicity in chicken gut microbiota

Eimeria infection has a severe impact on broilers resulting in known physical symptoms such as depressed growth performance, the decline in body weight gain, and low production (Liu et al., 2018; Zhou et al., 2020). These clinical effects remain leading contributor to economic loss, with poultry industry suffering a great loss in treatment and mortality of chickens. The expression of these clinical symptoms is due to internal disruption of enterocytes and intestinal epithelial cells, causing unevenness in intestinal homeostasis and elevated susceptibility risk of the host to other diseases (Hessenberger et al., 2016; Shang et al., 2016). During an *E. tenella* infection, the presence of severe intestinal epithelial injuries negatively influences the

proliferation and growth of resident bacteria (Chen et al., 2020). Cytokines such as IL-17A, IL-10, IL-1β and IFN-γ are secreted, providing pathways that favour proliferation and survival of pathogens (Wei et al., 2019). *Eimeria* uses these pathways (Toll-like receptors- TLR-2 and TLR 6) to exploit the IL-10 mRNA production, invade the host immune system and complete its life cycle (Sand et al., 2016). The high jacking of pathways by *Eimeria* pose a great threat to the health of chickens, affecting proper production and utilization of nutrients.

Eimeria infection destroys caecal tissues and intestinal lining structure, causing a disturbance in the gut microbial community, known as dysbiosis (Ducatelle et al., 2015). Dysbiosis is exhibited by a significant fluctuation of beneficial bacteria, while harmful bacteria accumulate to the extent of becoming a potential threat to the host, causing an imbalance of host's homeostasis (Cui et al., 2017). An overview of the impact of *Eimeria* infection on bacterial species of the gut is shown in Figure 1. Zhou et al. (2020) reported that E. tenella infection altered composition and diversity of caecal microbiota, significantly reducing *Proteobacteria* and *Firmicutes (Enterococcus)*. It also causes shifted gut microbiota, reducing the caecal microbial diversity of chickens (Wu et al., 2019). *Eimeria* infection affects the bird's ability to digest nutrients by reducing intestinal barrier function, causing bacterial translocation, affecting bacteria-dependent metabolic processes in the GIT (Shang et al., 2018). Latorre et al. (2018) reported that necrotic enteric diseases like coccidiosis affected bacterial diversity and composition in the GIT, reducing bacterial species such as *Firmicutes*, (mainly *Ruminococcaceae*) and SCFA-producing bacteria. *Eimeria* infection can decrease the frequency of immune-modulating bacteria (i.e., *Candidatus savagella*, *Ruminococceae*) while the abundance of bacteria (*Clostridium* spp.) that aggregate lesions and coccidia replication increases, causing severe damage to the mucosa (Hauck, 2017).

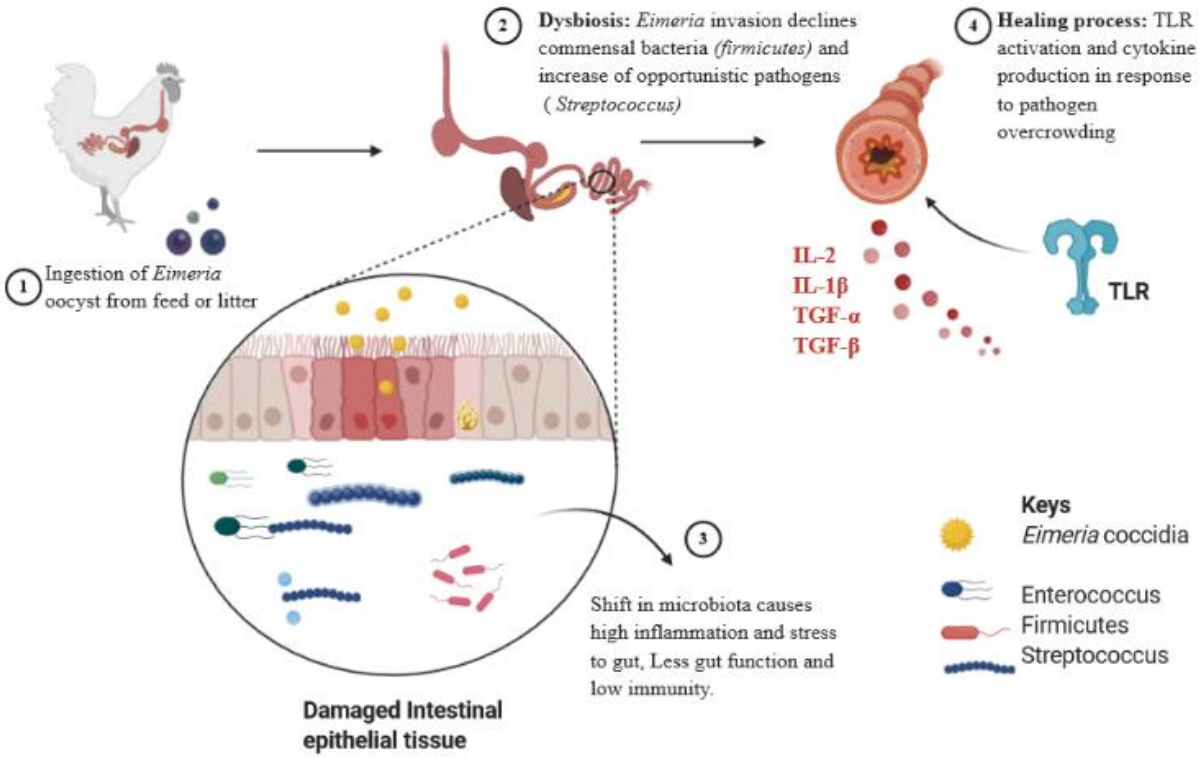

Figure 2.1: Eimeria infection on gut microbiota.

A study by Macdonald et al. (2017) examining the effect of *Eimeria* infection (*E. tenella*) on caecal microbiome diversity revealed that *Eimeria* infection did not affect microbial alpha diversity but induced significant changes observed by severe lesion score damage in the caeca and reduction in the microbial population of *Bacillales* and *Lactobacillales*, suggesting that fluctuations in abundance of the bacterial community may contribute to the severity of pathology and variation observed in tissue damage. Co-infections of *Eimeria* and *C. perfringen* also result in severe necrotic enteritis (NE) lesions with a significant increase of *Clostridium sensu stricto 1, Escherichia Shigella, and Weissella* and reduction in the *Lactobacillus* population located in the jejunum (Bortoluzzi et al., 2019; Wang et al., 2019). Reduction of commensal bacteria, such as *Firmicutes (Lactobacillus*) affects microbial diversity. It disrupts crucial metabolic processes that provide energy and carbon source of the host (Yan et al., 2017). These bacteria aid in converting glucose to lactic acid, lactate, acetic acid, ethanol, and CO2, which serves as an energy powerhouse for the host (Forte et al., 2018). *Proteobacteria (Campylobacter*) prevalence assists hydrogenases in hydrolysing indigestible sugars, namely, polysaccharides, oligosaccharides, and disaccharides aiding in the production of SFCAs (Bortoluzzi et al., 2018). Depletion of these bacteria due to *E. tenella* infection results in the build-up of complex compounds like uric acid and non-starch substrate indigestion. These

compounds can be toxic to the host, hindering the production of amino acids essential for facilitating the production of SCFAs absorbed by enterocytes (Clavijo and Florez, 2017).

Some reports show that *Eimeria* infection alone has little to no effect on α-diversity and caecal microbiota, co-infection of *Eimeria* and other predisposing factors (*C. perfringens*) of GIT can reshape or shift intestinal microbiota. Microbial shift results loss of diversity and depletion of crucial gut microbiota, e.g., *Lactobacillus*, while favouring overgrowth of pathogenic bacterial strains(*Clostridium, Salmonella* and *Weissella*) that affect growth and health of the birds (Hernandez-Patlan et al., 2019; Kubasova et al., 2019; Moore, 2016; Ocejo et al., 2019; Yang et al., 2019b). Supplementation of deficient bacteria to control *Eimeria* challenge is crucial, whether, by the administration of natural dietary feeds/additives such as probiotics, prebiotics, and phytochemicals has the potential of improving function, the abundance of microbes and modulating gut microbiota in GIT.

2.6. Dietary Supplementation for manipulating the chicken gut microbiota

Various strategies are currently being employed to combat the severity and spread of coccidiosis in chickens. Some of these control strategies include the use of live vaccines, therapeutic antimicrobial growth promoters (AGPs) and anticoccidial drugs (such as ionophore-based prophylactic drugs) produced from synthetic chemicals and fermentation methods (Awais et al., 2019; Jenkins et al., 2019; Oh et al., 2018). The use of these control strategies in previous years has proved effective in controlling the spread of *Eimeria* infections, however continuous exposure of animals to live vaccines and antibiotic growth promoters(AGPs) was discouraged as it poses negative impacts on food animals and indirectly public health concerns to humans (Yadav and Jha, 2019).

The use of live vaccines and AGPs (ionophores) hinders host colonization by parasites through disruption of the parasite's replication cycle, interference of ion transfer, and other biochemical pathways in the plasma membrane of the parasite, leading parasite death. Regardless of the effectiveness of these controls, continuous exposure of poultry to such treatments has resulted in traces of chemicals or compounds being left in poultry products (meat and eggs) as residuals that could have potential harm to humans when consumed, raising more concerning and importance of their discontinuation (Awais et al., 2019; Oh et al., 2018; Reis et al., 2018). As a result, a diversion is observed in research to explore various antibiotic-independent alternative strategies that are safe, antibiotic-free and cost-effective to ameliorate coccidiosis in poultry, e.g., the use of probiotics, prebiotics, organic acids, phytobiotics as diet supplements

in broiler feed and other alternative measures to prevent coccidiosis (Abbas et al., 2017; Gadde et al., 2017; Zheng et al., 2016). Coccidiosis greatly impacts gut barrier function, which is crucial for optimal health and defence of host; hence, an ideal feed additive should promote efficient barrier function of the gut. Chen et al. (2015) identified potential biomarkers (IL-8 and TGF-β) for barrier failure in broilers relevant for gut functioning in chickens. Administration of natural feeds such as probiotics should trigger cytokine production (IL-2, TGF-α) to confront coccidiosis.

2.6.1. Modulation of gut microbiota by probiotics against coccidial infection

Probiotics are live, well-defined non-pathogenic culture of microorganisms ingested by the host, crucial in improving inhabitant intestinal microbiota. These are often bacteria or yeast extracts added to the broiler's diet to enhance production and animal health by improving the intestinal microbial balance (Awais et al., 2019). These bacteria or extracts secrete crucial substances like bacteriocins and organic acids that contribute to health and homeostasis of the intestinal tract. They can protect against the invasion of pathogenic bacteria and coccidiosis by competitive exclusion (Chang et al., 2016; Hernandez-Patlan et al., 2019). Investigating probiotics as potential alternative strategies to alleviate *Eimeria* challenge in broilers is still crucial following previous literature reporting on the benefits of using these natural additives.

Probiotics have been reported to exhibit immunomodulation properties by manipulating and modifying gut microbiota, resulting in improved feed conversion ratio (FCR) and performance in broilers (Awais et al., 2019; Smialek et al., 2018). Some of the bacteria used as probiotics are isolated from fermented milk, including *Lactobacillus, Bacillus*, *Pediococcus*, Saccharomyces cerevisiae and Enterococcus faecium (Erdoğmuş et al., 2018). Li et al. (2017) reported effective response observed in broilers upon administration of Lactobacillus based probiotics (*L. acidophilus*), which boosted inflammatory response through production of acidophilin and modulated the immune responses (innate and acquired) of poultry, providing a favourable environment for beneficial bacteria. (Gharib- Naseri et al., 2019).

During *Eimeria* infection, the parasites replicate and adhere to receptors located in the intestines' epithelial surface. Intestinal compatible probiotic bacteria challenge *Eimeria* parasite in adhering to the intestinal mucosa and absorb receptors in the epithelial cells, preventing invasion (Jarujareet et al., 2018). This attachment hinders the perforation and secretion of *Eimeria* sporozoites into the intestinal mucosa, further resulting in reduced proliferation and oocyst shedding (Oyewole et al., 2018). This regulates the host's intestinal permeability,

improves barrier functions of the gut, and balances host gut microbiota (Huyghebaert et al., 2011). *Eimeria* infection affects intestinal permeability, so the administration of feed additive like probiotics promotes the balance of gut microbiota and regulates the barrier function to prevent the entry of foreign microorganisms in the GIT.

Probiotics improve beneficial bacteria's ability to adhere to the GIT, enabling them to occupy most of the tract while inhibiting the growth of pathogenic bacteria and parasites (Wang et al., 2019). Probiotics employ other competitive exclusion modes of action to reduce parasite colonization, including competition for sites of attachments, co-aggregation with pathogens and certain gut microbes and production of antimicrobial compounds that stimulate the immune system, e.g., lactic acid, hydrogen peroxide and bacteriocins (Alagawany et al., 2018; Kers et al., 2018). These mechanisms promote animal growth and balance in gut health through microbial abundance in the GIT (Sethiya, 2016). Competitive exclusion exhibited by probiotics against *Eimeria* species results in improved intestinal health through gut maturation and improved integrity, leading to improved feed digestion and absorption, hence higher body weight gain is often observed in chickens after probiotic treatment (Royan, 2019). Alagawany et al. (2018) reviewed the modes of actions exhibited by probiotics to hinder invasion and proliferation of pathogens such as *Eimeria* and *Salmonella*. The mechanism employed by probiotics involves the production of antibacterial substances that play a role competitively excluding unwanted and harmful bacteria from entering the gut (Alagawany et al., 2018; Kers et al., 2018). Probiotics enhance the host's immune responses by regulating T helper cells, conferring improved protection against the *Eimeria* challenge (Gu et al., 2019). Research has revealed probiotics as a promising alternative to antibiotics; however, probiotic incorporation is highly dependent on chicken's diet (optimal dose) and selection of appropriate strains of probiotic microbes to exhibit effective action against specific pathogens (Ducatelle et al., 2015).

Probiotics are also crucial for stimulating the proliferation of the intestinal epithelium, which regulates the mucosal barrier by mucin in the intestinal wall of broilers, lowering bacterial diversity (Erdoğmuş et al., 2018; Gu et al., 2019). The use of probiotics such as B. subtilis as dietary supplementation modifies gut microbiota composition by improving growth performance and nutrient digestion in broilers through an increased abundance of Bacteroidetes and other commensal bacteria such as *Rumminococcus* (Li et al., 2017). Similar findings were reported by Wang et al. (2019) where B. subtilis was confirmed to reduce microbial diversity in the caecum by altering microbial community and elevating predominant species. The

abundance of these bacteria aids in the breakdown of indigestible fibres, releasing substances such as butyrate and SCFAs, which are essential for extra nutrients and energy for broilers (Gharib- Naseri et al., 2019; Leung at al., 2018). Probiotics mainly target microorganisms in the small intestines, where peak nutrient absorption is observed (Wang et al., 2019). The presence of probiotics (i.e., *Lactobacillus*) in different sites in the GIT increases nutrient utilization, hindering intestinal colonization and intestinal lesions caused by *E. tenella* invasion (Royan, 2019). In evaluating the immune effect of infection in broilers, Awais et al. (2019) revealed that *Lactobacillus* and *Saccharomyces* based probiotics enhanced immunological and performance capabilities of broilers challenged with *Eimeria*. This property enables probiotics to protect against *Eimeria* infections, thereby reducing coccidiosis prevalence.

Lactobacillus based probiotics (LBPs) are known for exerting anticoccidial properties against *E tenella* infections (Awais et al., 2019, Chen et al., 2020). These probiotics exhibit growth-promoting effects that promote enhanced cellular and humoral immune responses, restricting the invasion of the GIT by *Eimeria* spp. (Leung et al., 2018). They also act as antagonists by producing cytokines and local antibodies (Immunoglobulin A-IgA), that are crucial for confronting coccidiosis, i.e., Interleukin-2 (IL-2), interferon-γ (IFN-γ) and IL-6 stimulate local cell-mediated immunity inhibiting invasion of E. acervulina (Behnamifar et al., 2019; Gharib-Naseri et al., 2019; Gu et al., 2019; Smialek et al., 2018). Cytokines are natural proteins essential for stimulation and regulation of immunity against infectious diseases. These substances also hinder the secretion of siderophores, restricting iron availability to facilitate parasite invasion (Awais et al., 2019). Probiotics also induce the production of which protect *Eimeria* by stimulating mucosal immunity and reducing oocyst shedding (Leung, 2018).

Chen et al. (2016) previously showed that a probiotic mixture containing four strains of lactic acid bacteria (*L. acidophilus, L. fermentum, L. Planetarium, and E. faecium*) was effective in reducing intestinal ulcers observed in broilers due to E. tenella infection and significantly influenced the level of expression of specific genes crucial for inflammation, e.g., cytokines (Gu et al., 2019). Lactobacillus based probiotics reduced levels of caecal gene expression of cytokines favouring inflammation (e.g., interleukin (IL)-1β and IL-6) and interferon (IFN)-γ, while anti-inflammation cytokine, i.e., anti-IL-10, was increased (Arendt et al., 2016, Chen et al., 2016). Probiotics' capability to exert stimulating effects on the immune system while altering the gut microbiota profile (Moore, 2016) cement their consideration as suited candidates to control coccidiosis. More research has been done to determine the effectiveness of other probiotic microflora against coccidiosis. *E. faecum* increased the abundance of

beneficial bacteria while also modulating the composition of intestinal microflora. *E. faecium* and *Pediococcus* supplementation on broilers infected by *E. acervulina* exhibited immunomodulatory effects through increased production of cytokines aiding in the modulation of anti-inflammatory cytokines and other immune mediators, including IL-1β, IL-6, IL-10, and IFN-γ, reducing the severity of intestinal lesions caused by the infection (Lee et al., 2014; Tarasova et al., 2010; Wu et al., 2019). Although in-depth information on anticoccidial properties of some probiotics was reported, efforts in exploring other microflora in the gut with potential to induce protection against NE infectious diseases is still required.

Even though probiotics effectively control coccidiosis, research has revealed that simultaneous inclusion of prebiotics with probiotics as poultry feed additives can enhance the viability of probiotic microorganisms through a synergistic effect (Chen et al., 2020; Leung et al., 2018). Pineda- Quiroga et al. (2019) showed that supplementation of synbiotics in broilers' diet conferred modulatory effect on microbiota while enhancing various pathways (i.e., starch and sucrose metabolism) without interfering with the biological role of caecal microbiota compared to when pre- and probiotics are administered independently. Similarly, the combination of pro- and prebiotics allows prebiotics to activate probiotics for effective modulation of the metabolic response in the gut against infections while maintaining gut microbiota integrity and inhibition of potential pathogens present in the digestive system (Slizewska et al., 2019). Muthamilselvan et al. (2016) further reviewed anticoccidial action of herbal remedies against coccidiosis, noting close interaction between prebiotics and probiotic microorganisms inhabiting the gut. Prebiotics are non-digestible feed additives that contain natural dietary fibres such as fructooligosaccharides (FOS), inulin, β-glucans, and Mannooligosaccharides (MOS), which are crucial in inducing development or activity of commensal bacteria in the gut, excluding invasion of the harmful pathogens (Gadde et al., 2017; Nopvichai et al., 2019; Ocejo et al., 2019). Accumulation of gut probiotics mediated by prebiotics suppresses pathogens and significantly improves poultry's immune responses (Yadav and Jha, 2019). Even though *Eimeria* infections drastically reduce majoring of commensal bacteria, studies have found a mild increase of some *Firmicutes* (*Ruminococcaceae*) in infected chickens after *E. tenella* infection (Chen et al., 2020). Microbe recovery after infection allows fast restoration of GIT intestinal mucosa, maintaining gut homeostasis.

2.6.2. Stimulation and stabilization of gut microbiota through phytobiotics

The use of plant-based phytochemicals was associated with their antimicrobial and antiparasitic properties that enhance protective immunity in livestock infected by coccidiosis and other NE diseases (Idris et al., 2017). Phytobiotics include various products synthesized and extracted from plants such as herbs, essential oils, and oleoresins (Mohammadi Gheisar and Kim, 2017). Supplementation of these natural products as animal feeds was favoured because they are safe, cheap, readily available in nature and effective against various diseases (Abbas et al., 2017; Oh et al., 2018). Some of these natural foods and herbs improve immunity and animal's resistance to disease. The mechanisms of action utilized by phytochemicals in controlling infections include the interference with the life cycle of *E. tenella* by hindering oocyst sporulation, invasion of sporozoites and the maturation of schizonts (Yang et al., 2019b). They have been reported to impair *Eimeria* spp. in early development stages by reducing cell wall degradation and inducing oxidative stress, preventing invasion (Felici et al., 2020; Muthamiselvan et al., 2016). Similar findings were reported by Jiao et al. (2018) where administration of *Artemisia annua (A. annua*) and *Artemisinin* was confirmed to interfere with the *Eimeria* life cycle by producing reactive oxygen species that are effective in inhibition of oocyst sporulation and formation of the parasite cell wall.

Research done to date on phytonutrients such as *Capsicum annuum* (pepper), *Curcuma longa* (turmeric), *Lentinus edodes* (shiitake mushroom), *Carthamus tinctorius* (safflower) revealed promising inhibitory properties and defence mechanisms against infections in chickens, promoting enhanced gut health and immune system (Kim et al., 2015; Wagle et al., 2019). There is a wide range of commercialised plant-derived compounds available, and their effect on chicken health thus far has been promising. Some phytochemicals and their effect on immune response and gut health are presented in Table 1. Administration of phytochemicals in chickens challenged with *Eimeria* alters and stabilises the intestinal microbiota by shifting the composition of beneficial bacteria and reducing microbial metabolites in the gut, improving gut health (Yang et al., 2020). Kim et al. (2015) further confirmed that the administration of *Capsicum* and curcumin longa oleoresins affected microbial population by increasing abundance of *Lactobacillus* and OTUs while reducing the population of *Selenihalanaerobacter* in two chicken breeds (Ross and Cobb). Cinnamaldehyde and Oleoresins (Capsicum and Turmeric) have been reported to regulate host immunity against E. tenella through elevation of T helper cells and cytokines (FN-γ and IL-6) and body weight gain in poultry (Lee et al., 2020). A combination of curcuma and Capsicum exert synergistic effect towards coccidiosis by

enhancing innate immunity (Lillehoj et al. 2018). The mechanisms exhibited by phytobiotics on the regulation of the life cycle of *Eimeria* and growth regulation of gut bacteria makes phytochemicals best suited for supplemented in poultry diet at an early stage of development.

Table 2.1:Different phytochemical additives against coccidiosis in poultry

Phytochemical(s)	Effect on gut health and immune response	Reference
Areca Nut (*Areca Catechu L.*)	- Reduced caecal lesion scores - Enhanced immunity through the production of interleukin 2 (IL-2)	Wang et al., 2018
Bidens Pilosa	- Disrupt the life cycle of *E. tenella* - Enhance T-cell mediated immunity	Chang et al., 2016; Chen et al., 2020; Yang et al., 2015; Yang et al., 2019
Carvacrol	- Increased growth performance and intestinal barrier function - Alters gut physiology - Interference with the life cycle of *E. tenella* by destroying sporozoite membrane	Felici et al., 2020; Lee et al., 2020
Curcumin	- Maintains gut integrity - Interference with life cycle by inhibiting *E. tenella* sporozoites	Burt et al., 2013; Yadav et al., 2020
Cinnamaldehyde	- Improve chicken growth performance and alter caecal microbiota composition	Yang et al., 2020
Garlic (*Allium Sativum*)	- Increase resistance to experimental *Eimeria acervulina* infection - Induce alterations in broiler intestinal microbiota	Pourali et al., 2014; Sheoran et al., 2017

Similar results were detected by Chowdhurya et al. (2018) when evaluating the effect of three essential oils, i.e., cinnamon bark oil (CNO), clove bud oil (CLO) and ajwain seed oil (AJO),

where an abundance of *Escherichia coli* in pre-caecal contents decreased in groups given CNO but did not affect *Lactobacilli* spp. in any diets. Based on a range of activities exerted by phytobiotics in chickens, it can be suggested that the primary mode of action for most phytobiotics focuses on altering the gut environment and intestinal morphology, providing protection and enhanced resistance to infections when fed to healthy poultry (Idris et al., 2017; Jitviriyanon et al., 2016). From the findings mentioned above, the simultaneous use of feed additives may be beneficial to the host by providing different functions such as maintaining chicken gut health and targeting different aspects of animals' physiology to boost antimicrobial activity against diseases. Research has also shown that combined supplementation of phytochemicals exerts boosted anticoccidial effect in broilers compared to when administered on their own (Felici et al., 2020; Sidiropoulou et al., 2020).

2.6.3. Recombinant vaccines as a promising alternative preventive option

The inclusion of recombinant DNA vaccines has shown promising consequences in chicken welfare against coccidiosis by induction of protective immunity (Sander et al., 2019). Recombinant vaccines consist of *Eimeria* gene encoding protective antigens that interact with both host and parasite through parasite invasion processes. These antigens are associated with parasite surface and internal proteins secreted by organelles such as rhoptries and micronemes (Ding et al., 2004). The use of recombinant vaccines, whether alone or in combination with other alternatives confers similar effective immunoprotection to host. However, some setbacks have been reported where DNA vaccines fail to induce immunogenic response (cellular immunity) in animals (Ding et al., 2016). Recent studies have looked at combination recombinant vaccines with natural additives for enhanced protection against *Eimeria*. Ding et al. (2004) reported that immunization of chickens with recombinant *E acervulina* (3- 1E) vaccine and cytokine genes reduced oocyst shedding and increased body weight gain and enhanced protective intestinal immunity against *E. acervulina* challenge. Similar findings were observed by Ritzi et al. (2016) who evaluated the effect of a combination of probiotic and vaccine treatment in Cobb 500 broilers challenged with *Eimeria*, where combined treatment enhanced protective effect against the challenge by lowering lesion score and improving the performance of chickens. With in-depth public research elucidating probiotics' effects on gut microbiota, the effective consequences observed with combination treatments encourage further exploration of recombinant vaccines interaction with the chicken gastrointestinal tract.

2.7. **CONCLUSION AND FUTURE RECOMMENDATIONS**

Coherent interaction between gut microbiota and the host is crucial for the normal functioning and health of poultry. Presence of a coccidian infection (i.e., *Eimeria* or *Isospora*) in chicken compromises gut microbiota resulting in the imbalance of the microbial communities, affecting their active role within a host. Supplementation of natural alternatives such as probiotics and phytochemicals remain the most favoured strategy currently to combat coccidiosis, without the effect of drug resistances threatening biosecurity. Manipulation of gut microbiota by introducing natural additives at an early stage of development may improve the immune system of chickens against *Eimeria* infections. Since these additives are chemicals and strains fermented by bacteria, they can persist in the GIT as a beneficiary gut microbiome for a long time, without posing any adverse effect on broilers. Thus, the use of natural alternatives (feed additives) is recommended for fast GIT restoration and modulation of gut microbiota in response to coccidian challenges while improving growth performance and chickens' overall health. The simultaneous inclusion of recombinant vaccines and natural additives has expressed potential boosted immunomodulatory function of gut microbiota while also providing enhanced immunoprotection to host, critical for evading *Eimeria* challenge in poultry. Significant progress has been made in discovering alternative strategies to combat coccidiosis; however, further studies focusing on mutual interaction of feed additives with host gut microbiota towards *Eimeria* challenge are still required to provide efficacious treatment measures. Combination of recombinant vaccines and natural products like probiotics or phytochemicals as immunogenic adjuvants through immunoinformatics approach may transform the discovery and development of vaccines. However more research is needed to confirm.

Author contributions

T Madlala wrote the manuscript. M Okpeku and MA Adeleke conceptualised and supervised the work and corrected the manuscript. All authors approved the manuscript.

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Declaration of conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER THREE

Designing T-cell epitope-based vaccine against *Eimeria* from Immune mapped protein 1 (IMP-1) antigen using immunoinformatic approach

3.1. ABSTRACT

Drug resistance against coccidiosis disease has posed a significant threat on chicken welfare and productivity worldwide, putting daunting pressure on the poultry industry to reduce the use of chemoprophylactic drugs and live vaccines in poultry as treatment and prevention methods of the intestinal disease. Despite their effectiveness over the years, their continued administration was noted to result in drug resistance in parasites and the presence of drug residues in food threatening food security. Chicken coccidiosis, caused by an apicomplexan parasite of *Eimeria* spp., is currently a significant challenge worldwide. Due to the experience of economic loss in production and prevention of the disease, the development of cost-effective vaccines or drugs that can stimulate defence against multiple *Eimeria* species is imperative to control coccidiosis. This study explored *Eimeria* immune mapped protein-1 (IMP-1) to develop a multiepitope-based vaccine against coccidiosis by identifying antigenic T-cell epitope candidates through immunoinformatic techniques. This resulted in the design of 7 $CD8^+$ and 21 $CD4^+$ T-cell epitopes, which were connected together using AAY and GPGPG linkers to form a vaccine construct. A Cholera Toxin B (CTB) adjuvant was attached to the N-terminal of the multiepitope construct to improve the immunogenicity of the vaccine. The designed multi-epitope vaccine was assessed for its immunogenicity (10.152), allergenicity and physiochemical parameters, which revealed the construct molecular weight of 60.87 kDa, theoretical pI of 6.33 and instability index of 31.70. Protein-protein docking simulation of vaccine with TLR-5 revealed good structural interaction and stability of protein structure of vaccine construct, with binding affinity of -159.964 kcal/mol. The designed vaccine predicts the induction of immunity through production of antibodies and cytokines, which may be vital in hindering the surface entry of parasite into host while boosting the host's immune system, though further experimental study is still required to validate this.

Keywords: Coccidiosis, *Eimeria*, Vaccine, IMP-1, Immunoinformatic, Immune response

3.2. INTRODUCTION

The impact of coccidiosis on poultry production , and the evolution of drug resistance to known prophylactic drugs, attenuated and non-attenuated vaccine, is currentlyfelt globally, and it is continuously inflicting grave economic loss to the poultry industry (Chen et al., 2020; Song et al., 2015). Avian coccidiosis is a ubiquitous intestinal disease caused by *Eimeria* species, which are intracellular obligate Apicomplexan protozoans (Boulton et al., 2018; Shirley., 2000). This disease is characterized by the gut epithelial cells invasion by Eimerial sporozoites, resulting in clinical symptoms such as malabsorption and increased vulnerability to other pathogen infections (Chapman, 2014; Soutter et al., 2020).

Eimeria parasites consist of fairly large genomes with their size ranging from 55 and 60 Mbp, carrying 8,000 to 9,000 genes (Tang et al., 2020). The classification of Eimeria genomes includes the nuclear genome, which carries approximately 60 Mbp of DNA within 14 chromosomes of 1–7 Mb (Shirley, 2000). Other genomes are the mitochondrial genome of ~6200 bp, a circular apicoplast genome comprising ~35 kb circular extrachromosomal DNA and a double-stranded RNA viral genome (Blake et al., 2020). This genome composition is the most complex and prone to evolve rapidly, due to the fast life cycle of *Eimeria* (Cai et al., 2003). *Eimeria* infection displays a direct faecal-oral life cycle, where the chickens ingest sporulated oocysts from polluted litter and feeds (Hauck, 2017). This infection can be visibly detected in chickens through clinical symptoms like the intestinal inflammation, leading to haemorrhage and bloody diarrhoea. The clinical symptoms of this disease cause infected chickens to be vulnerable to other pathogens such as *Clostridium perfringens*, significantly compromising poultry's performance and productivity resulting in high mortality (Reid et al., 2014; Soutter et al., 2020).

Over the years, the control of *Eimeria* in chicken production has heavily been dependant on the use of prophylactic chemotherapy and various vaccines containing components of live virulent or attenuated *Eimeria* as an effective mode of control (Song et al., 2015). However, the continuous administration of these anticoccidials has triggered the occurrence of drug resistance in parasites and compromised quality of meat or egg production with traces of chemical residues detected in food, threatening food security (Soutter et al., 2020; Tang et al., 2018). Production of these vaccines and anticoccidial drugs require high cost for their continued production (Yan et al., 2018). This has urged the advancement of novel strategies to control and prevent the disease. The advancement on developing novel and cost-efficient

vaccines against *Eimeria* is highly imperative to manage coccidiosis. The use of immunoinformatic and reverse vaccinology on the existing knowledge about *Eimeria* species and the complexity of their life cycle has brought a paradigm shift in vaccine development, with most research shifting its focus to antigens involved in host/parasite interaction and in the developmental stages of the parasite.

Immune mapped protein-1 (IMP-1) is a membrane protein that is highly conserved, and a protective antigen discovered in *E. maxima* (Blake et al.,2011; Tang et al., 2018). It was identified as an immunogenic and surface-expressed antigen with the ability to confer protection against *Eimeria* infections and from other apicomplexan parasites such as *Toxoplasma* (Yin et al., 2013). Various studies have shown that vaccination with formulations expressing IMP-1 from *E. maxima* using different vectors as a delivery vehicle, induces significant protection against Eimeria challenge (Pastor-Fernández et al., 2020). Literature has reported on the design and construction of multi-epitope DNA vaccines and has confirmed efficacy and safety of these vaccines against numerous pathogens such *Plasmodium falciparum* (malaria), *Bunyavirus (*Rift Valley fever virus*), Klebsiella Pneumoniae, Toxoplasma gondii* and *adenovirus serotype 4 (FAdV-4)* (Adhikari and Rahman, 2017; Aziz et al., 2019; Dar et al., 2019; Pandey et al., 2018). Construction of multi-epitope vaccine also plays a crucial role in ongoing, ground-breaking research to find novel strategies to minimize the Corona Virus pandemic currently faced globally caused by SARS-CoV-2 (Dong et al., 2020; Kar et al., 2020; Samad et al., 2020; Singh et al., 2020). Hence this study explored *Eimeria* IMP-1 antigen with the aim to develop a potentially cost-effective multiepitope-based vaccine against *Eimeria* by identifying antigenic T-cell epitopes through immunoinformatic techniques and evaluating the effectiveness of the identified epitopes in inducing immune response within the host through the construction of a multi-epitope vaccine.

3.3. **METHODS AND MATERIAL**

3.3.1. The retrieval of Eimeria protein sequences and identification of preserved sequences from the genomic sequences

The protein sequences of *Eimeria* Immune mapped protein 1 (IMP1) antigen were retrieved from the protein database of the National Centre for Biotechnology Information- NCBI[1].

The obtained sequences were subjected to multiple sequence alignment (MSA) with conserved CD4 and CD8 chains capable of inducing immunological responses. MSA was performed using the CLUSTALW online server[2] (Thompson et al., 1994). The MSA was performed using

default parameters and obtained results of conserved regions that matched genome sequence and conserved chains, which had a minimum of 15 amino acid residues were selected for further analysis.

3.3.2. Antigenicity and prediction of transmembrane of conserved regions

In this study, the selected conserved regions were tested for antigenicity using the VaxiJen v2.0 Server[3], with the threshold set to 0.4 (Doytchinova et al., 2007). The sequences identified as probable antigens were selected and tested for transmembrane helix properties in the TMHMM v2.0 server[4]. The TMHMM server was used to identify outer membrane protein sequences.

3.3.3. Identification of T-cell epitopes.

3.3.3.1. Prediction of cytotoxic T-cell / $CD8^+$ T-cell epitopes.

The preserved sequences that fulfilled the transmembrane analysis were submitted to the NetCTL v1.2 tool, to generate nonamers that can bind to major histocompatibility complex (MHC) class I HLA alleles molecules and induce $CD8^+$. The resulting nonamers were subjected to the IEDB analysis tool[5] to determine cytotoxic T-cell (CTL) epitopes (Andreatta and Nielsen, 2016). The nonamers were analysed using the Stabilized Matrix Base Method (SMM). The parameters for identifying MHC-I binding alleles selected included the amino acid length of peptide set to 9.0, the IC_{50} value< 250, and the human as MHC source species. Since there is no data currently available in the immunoinformatic tool for chicken MHC alleles used for MHC-epitope binding prediction, the human HLA alleles were selected to substitute the chicken MHC (Valdivia-Olarte et al., 2015; Aziz et al.,2020). Studies have showed that anchor residues in chicken BF haplotypes are similar to residues anchored on mammalian MHC-I especially those with 8-9mer in size (Tan et al., 2016). The generated epitopes were examined for antigenicity, with a threshold set to 0.5 as the main parameter. Sequences that were detected to be above the set threshold were selected as probable antigens. The CTL cell epitopes were further tested for immunogenicity using the MHC I immunogenicity tool of IEDB[6]. The immunogenicity tool was mainly used to identify sequences that can stimulate an immune response towards any parasites in a host (human or animal) (Adhikari et al., 2017).

3.3.3.2. Helper T-cell/ $CD4^+$ T-cell epitopes epitope prediction.

The helper T-cell (HTL) epitopes were predicted using the IEDB MHC II binding tool[7]. The prediction method used was SMM-align (stabilization matrix alignment), allele length was set at 15, and the threshold for IC_{50} was < 250 (Nielsen et al., 2007). This technique also allowed identification of MHC class II HLA alleles that bind to HTL epitopes. The identified HTL epitopes were subjected to the IFNepitope tool[8] to predict epitopes that could induce cytokine IFN-γ. The method and parameters used for prediction was the SVM (support vector machine) based method and the IFN-γ versus non-IFN-γ model. The identification of IL-4 inducers was achieved through the IL4pred tool[9]. The resulting shortlisted epitopes were scrutinized using similar immunoinformatic tools as CTL epitopes, to determine their antigenicity with a threshold set at 0.5.

3.3.4. Conservancy and Allergenicity test

The generated T-cell epitopes identified as antigenic and immunogenic were tested for conservancy using IEDB conservation across antigen tool[10] (Bui et al., 2007). The allergenicity of the conserved epitopes was determined by AllerTop v2.0 tool, where sequences identified as allergens were discarded, and only non-allergic sequences were selected (Dimitrov et al., 2013).

3.3.5. Tertiary modelling of the $CD8^+$ and $CD4^+$ T-cell epitopes

The selected epitopes were docked with chicken MHC alleles to identify binding interaction between $CD8^+$, $CD4^+$ T-cell epitopes and the MHC-alleles and stability of the proteins. The chicken MHC-alleles- BF2*2101 (pdb: 4D0C) and pBL2*019:01 (PDB: 6KVM) were recovered from the RCSB Protein Data Bank (PDB)[11]. Before docking, the chicken alleles were first prepared using naccess tool[12] where the active and passive residues were generated and later viewed in chimera v1.14[13] (Pettersen et al., 2004). Molecular docking of epitopes with PDB structure of the binding MHC alleles was performed using the ATTRACT Peptide online[14] to generate haddock files to be used to complete docking. The prepared MHC-allele structures with haddock files from AttractPep were further subjected to the CHPC platform to complete docking simulation on the locally installed attract, to obtain structural flexibility. A total of 50 models were generated for each epitope. The model with the lowest binding energy and which

properly occupied the receptor was selected as the best-docked model from the generated models. The selected best-docked models were then combined to form one epitope-complex, and their structural illustrations were visualised using Chimera[13].

3.3.6. Epitope merging for generation of multi-epitope subunit vaccine

The prioritized epitope candidates for $CD8^+$ and $CD4^+$ were determined using various immunoinformatic tools were joined together with an immunological adjuvant to form multiepitope vaccine. The $CD8^+$ T cell epitopes were joined together with an aid of AAY linkers, $CD4^+$ T cell epitopes were linked by GPGPG linkers, and an appropriate adjuvant was attached to the N-terminal of the vaccine with the aid of the EAAK linker. These linkers provide extended flexibility to the peptides making up the vaccine, making it more stable. The addition of the adjuvant to the vaccine is crucial as it boosts the immunogenicity of the multiepitope construct (Dong et al., 2020). The adjuvant added to the selected T-cell epitopes was a cholera toxin subunit B (CTB) sequence (accession no. ABV74245.1).

3.3.7. Antigenicity, allergenicity, solubility, and physiochemical properties assessment

The antigenicity of final multiepitope vaccine sequence was tested using Vaxijen v2.0 server[15]. The predicted vaccine's antigenic nature ensured its ability to bind and interact with the receptor during the docking stage. AllerTop v2.0 was used to further determine the constructed vaccine as an allergen or non-allergen. To assess physicochemical parameters of the vaccine protein, the vaccine sequence was subjected to ProtParam53 web server[16] from the Expert Protein Analysis System (EXPASY) to calculate the number of amino acids of the vaccine, molecular weight (kDa), theoretical isoelectric point (pI), estimated half-life, instability index, aliphatic index, hydropathicity GRAVY (Gasteiger et al., 2005).

3.3.8. Tertiary structure prediction, refinement, and validation

The tertiary structure of the previously designed multiepitope vaccine protein was predicted and generated using the RaptorX server[17]. Since the designed vaccine was a novel protein without any known template, Raptor was the best suited for structural predictions. Molecular refinement of the predicted vaccine tertiary structure was achieved using the GalaxyRefine server[18] (Heo et al., 2013; Singh et al., 2020). The structure refinement was done to improve the structural quality of the vaccine protein. The GalaxyRefine server predicted five refined models of the vaccine construct resulting from structural perturbations and structural relaxations. From the refined models, model 1 was predicted by structure perturbation applied

to the clusters of the side chains and models 2-5 were generated by more aggressive perturbations (Ko et al., 2012). All the five refined models were further checked for GDT-HA, RMSD, MolProbity score and the best-refined model was selected and validated. The validation of the selected refined tertiary structure for the designed vaccine protein was performed using PROCHECK, which generated a Ramachandran plot and ProSA-web[19] was employed for final validation which generated a Z-score for confirmation (Laskowski et al., 1993).

3.3.9. Molecular docking of vaccine constructs with Toll-like receptor 5.

Molecular docking of designed multiepitope vaccine with toll-like receptor (TLR5) was performed using AttractPep and completed using locally installed attract package from the Centre of High-Performance Computing (CHPC) Lengau cluster, with final model visualizations obtained from Chimera. Docking of vaccine with TLR5 was done to check the vaccine's binding affinity and agonistic ability towards receptor molecule. To start docking, Solvent accessibility was calculated with Naccess tool to access the active and passive residues of both the vaccine construct and TLR5, which were then used in docking simulation and later viewed in chimera v1.14. The PDB structure of TLR5, active residues of receptor and sequence of the multiepitope vaccine were submitted to AttractPep (DeVries et al., 2017). Results obtained and the refined structure of the vaccine construct were further subjected to CHPC Lengau cluster to complete the docking with locally installed ATTRACT. A total of 50 models were generated for the multiepitope vaccine. The model with the lowest energy and binding properly to the receptor was selected from the generated models and visualized using VMD server. Chimera was used to visualize the best-docked tertiary structure of the refined TLR-vaccine.

3.3.10. Molecular dynamics simulations and analysis

Docked complex of multiepitope vaccine and TLR5 was further subjected to energy minimization through molecular dynamics simulations (MDS) using AMBERS 14 packages (literature et al., 2014). MDS was performed to evaluate complex stability and interactions between the docked proteins (Yadav et al., 2020). To minimize high energy configurations in the protein, energy minimization was performed. It was initially performed with 1000 steps (500 steepest descents with 9500 conjugate gradient) and followed by full minimization of 2000 steps. The density of the water was controlled with 4 ns while equilibration was 2 ns at 300 K. The MDS production was run for 100 ns. Data analysis of parameters Root Mean Square

Fluctuations (RMSF) and Root Mean Square Deviation (RMSD) was performed, and results were graphed.

3.3.11. In silico codon optimization, cloning and expression of vaccine construct

Codon optimization of multiepitope construct was achieved using Java Codon Adaptation Tool (JCat)[20] (Grote et al., 2005). Optimization was performed to obtain improved nucleotide sequence adapted to its potential selected expression host (E. coli strain K12). The JCat adaptation is dependent on codon adaptation index (CAI) and percentage GC content of improved sequence. The optimal CAI score of optimized gene sequence ranges from is 0.8-1.0 and GC% (30-70%), indicating improved expression of gene in its corresponding organism, without any translation errors (Yadav et al., 2020). The optimized nucleotide sequence of vaccine was cloned and expressed in *E. coli* (strain K12) host, where XhoI (CTCGAG) and BamHI (GGATCC) restriction sites were added to 5'and 3' ends of the construct prior cloning, respectively. To clone the improved vaccine sequence into suitable expression vector, SnapGene software was used.

3.3.12. *Immune simulation*

The multiepitope peptide was further subjected to an online *in silico* immune simulation server, the C-ImmSim server[21] to generate and evaluate vaccine candidate immune response (Rapin et al., 2010). All simulation parameters used for simulation were set at default (Table S3.1) three vaccine administrations given at intervals of four weeks (Shey et al., 2019).

3.4. RESULTS

3.4.1. The retrieval of Eimeria protein sequences and identification of preserved sequences from the genomic sequences.

To design the multi-epitope subunit vaccine, a total of 19 *Eimeria* Immune Mapped protein-1 antigen genomic sequences representing all *Eimeria* species were retrieved from NCBI. The genome's conserved sequences were created through multiple sequence alignment using the CLUSTALW online server, where 22 unique and conserved sequences were selected.

3.4.2. Antigenicity and prediction of transmembrane of conserved regions.

All the selected conserved sequences were tested for antigenicity with default parameters of the threshold value set at ≥0.4, in Vaxijen v2.0 server. It was found that 16 sequences fulfilled the antigenicity property of set threshold with Vaxijen score ranging from 0.4295 to 0.9046 (Table 3.1).The transmembrane analysis performed using TMHMM v2.0 server detected about 12 conserved sequences that fulfilled and exhibited the exomembrane properties (Table 3.1).

Table 3.1: Conserved sequences of genome sequence of *Eimeria*

Conserved sequences	**Antigenicity Score**	**Transmembrane helix**
AEEIENKVLPVKEEDAFNISAFGFVAVTPPPPPYKAGANITPKRFGEIATGAGGAYLQLS	0.4295	outside
GITYFLQEMKYKWEVWSKVQRQAYYQGWIKFVKAADEMEASFTLHHFAAPAPPAKLFLLH	0.5288	outside
GKNELIRNLQSDKKLFYSGICQFVKEAKDIKGKLTLLQHFDSSFPIKVDLYF	0.5931	outside
GVTCLLQEMKYKWDVWSKVQRQPYYQGWMKFIKAADEMEASVKVHQFTSPAPAAKVFLLH	0.5782	outside
MGAACGKSQRAAAAVEPPLSTAEKAEAAAVAAAEHSQKAEEAAEVAAACATK	0.7171	inside
MGAACMKSHGAATDAVAPRRSTAEKAADAAAAAEEHSHKAQEAAETAAACARR	0.7931	inside
MGAACMKSHIGPRSAAEAACPPTAAEKAAEAASEAAIHSGKPEEAEEAAAAAAEAPGAAV	0.6764	outside
MGAACMKSQGGATPPAAGGVSAAQKVAEATAAAAEHSMKAHQAAETAAACAKR	0.7150	Inside
MGAACTKSKGTAAPAARPSTTDRATEAAAAAAEHSQKAQEAAEEAIACAAK	0.7889	Inside
MGGACGKSRGTAAAAAAAPPVSAADKAAEAAASAASHAEKAQEAAAAAAAAAAN	0.9042	outside
MGGACGKSRGTAAAAEPPVSAADKAAEAAASAASQAEKAQEAAAAAAAAAAN	0.9046	outside
SGPIENKVLPVMEEESFSVSVFGFAAVTPPPSPYKAGANISPKRFGEIATAAGGGYVQLS	0.6084	outside
TGPGGPTYSFLAEGGMLHLMKPRCFCLMFLALKWD	0.6811	outside
TGPIENKVLPAKEEEPFNVSVFGLAAVTPPSPPYKPGANITPKRFGEIATGAGGAYMQLS	0.5831	outside
TGPIENKVLPVKEEEEFKISVFGFAAVVPAQSSYKPGANITPKRFGEIATEAGGAYIQLS	0.7513	outside
TGPIENKVVPVKLGEPIGISMFGFAAVAPPPAPYKAGANITPKRFGELATQAGGAYIQLS	0.4525	outside

3.4.3. *Prediction of T-cell epitopes*

3.4.3.1 Prediction of cytotoxic T- cell / $CD8^+$ T-cell epitopes

The preserved sequences selected were subjected to the NetCTL v1.2 server, where a total of 577 receptor-specific immunogenic nonamers of CTL epitopes were found. The nonamers were subjected to the IEDB MHC-I prediction tool, where the SSM-based method and the IC_{50} value parameter < 250 were set to predict MHC-I binding alleles accurately. The prediction analysis detected that about 214 CTL epitopes interacted with one to eight MHC alleles under the set IC_{50} parameter. The selected epitopes were further tested for antigenicity with a threshold value set at ≥0.5, in Vaxijen v2.0 server. A total of 77 CTL epitopes were detected to be antigenic in nature with epitope 'FKISVFGFA' having the highest Vaxijen score of 2 .2931. The immunogenicity analysis performed using the IEDB tool identified 41 sequences as immunogenic. These epitopes were tested for conservancy, where 20 epitopes were noted to be conserved. The conserved epitopes also underwent allergenicity analysis using AllerTop v2.0 server, where seven (7) $CD8^+$ epitopes: AQEAAAAAA, EAAAAAAAA, FGFVAVTPP, FKISVFGFA, FNISAFGFV, FTSPAPAAK and KISVFGFAA were found to be non-allergens and were regarded as final predicted epitopes. The summary of results obtained for final predicted $CD8^+$ T cell epitopes, IC_{50}, antigenicity, immunogenicity and allergenicity scores are presented in Table 3.2.

Table 3.2: Final predicted CD 8^+ T cells epitopes, 100 overlapped with $CD4^+$T cell epitopes and interacting with different MHC 1 alleles

Epitopes	HLA alleles	IC_{50}	Antigenicity	Allergenicity
AQEAAAAAA	HLA-A*02:06	137.26	0.8280	Non allergen
EAAAAAAAA	HLA-A*35:01	124.33	0.8030	Non allergen
	HLA-A*68:02	34.087		
FGFVAVTPP	HLA-A*02:06	199.779	0.6274	Non- allergen
FKISVFGFA	HLA-A*02:06	139.49	2.2931	Non- allergen
FNISAFGFV	HLA-A*68:02	34.80	1.4278	Non- allergen
	HLA-A*02:06	57.22		
	HLA-A*02:03	227.48		
FTSPAPAAK	HLA-A*68:01	35.11	1.0977	Non- allergen
	HLA-A*11:01	158.23		
KISVFGFAA	HLA-A*02:06	228.32	1.4171	Non- allergen

3.4.3.2. Prediction of helper T-cell/ $CD4^+$ (HTL) epitopes

The 12 conserved sequences that previously fulfilled the transmembrane analysis were subjected to the IEDB MHC-II binding tool to predict HTL epitopes and their respective HLA alleles. The VaxiJen server was applied to the detected HTL epitopes to test for antigenicity. A total of 103 epitopes were obtained that fulfilled both the IEDB tool and Vaxijen parameters of IC_{50} value < 250 and ≥ 0.5 antigenicity score were considered as potential HTL epitopes. The identified HTL epitopes were assessed for conservancy, where 45 $CD4^+$ T- cell epitopes were selected as conserved. The identified conserved epitopes were further subjected to IFNepitope and IL-4pred immunoinformatic tools to identify HTL epitopes that can induce immune response through production of signal cytokines, i.e., IFN-gamma inducers and interleukin inducers. A total of 25 $CD4^+$ T-cell epitopes exhibited both IFN-gamma and IL-4 inducer properties, enhancing the immunogenic capacity of the potential vaccine. These epitopes were subjected to AllerTop v2.0 for allergenicity analysis, where the allergenicity analysis revealed 21 epitopes to be non-allergenic, making them final suitable predicted HTL epitopes candidates for vaccine development since they do not cause any allergic reactions to the host (Table 3.3). The epitope 'KEEEEFKISVFGFAA' with the highest antigenic VaxiJen score of 1.5250 was disregarded as the allergenicity analysis identified it as allergic.

Table 3.3: Final predicted $CD4^+$ T-cells epitopes, 100 overlapped with $CD8^+$ T cell epitopes and interacting with different MHC II alleles

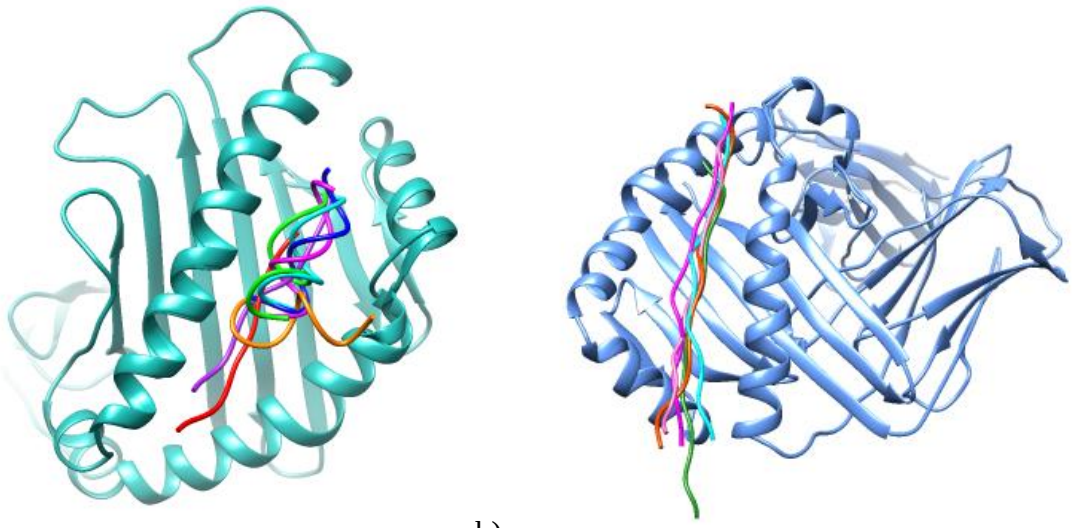
Epitopes	HLA alleles	IC_{50}	Antigenicity	Allergenicity
KAQEAAAAAAAAAAN	HLA-DRB1*01:01	235.00	0.8516	Non- allergen
AEKAQEAAAAAAAAA	HLA-DRB1*01:01	232.00	0.8224	Non- allergen
EKAQEAAAAAAAAAA	HLA-DRB1*01:01	230.00	0.8188	Non- allergen
AFNISAFGFVAVTPP	HLA-DRB1*04:04	248.00	0.9478	Non- allergen
AYYQGWIKFVKAADE	HLA-DRB1*04:04	62.00	0.6862	Non- allergen
	HLA-DRB1*04:05	119.00		
DAFNISAFGFVAVTP	HLA-DRB1*09:01	155.00	0.7499	Non- allergen
	HLA-DRB1*01:01	221.00		
EDAFNISAFGFVAVT	HLA-DRB1*09:01	152.00	0.6700	Non- allergen
	HLA-DRB1*01:01	204.00		
EEDAFNISAFGFVAV	HLA-DRB1*09:01	154.00	0.5405	Non- allergen

	HLA-DRB1*01:01	209.00		
EEEEFKISVFGFAAV	HLA-DRB1*09:01	234.00	1.1886	Non- allergen
	HLA-DRB1*01:01	194.00		
EEEFKISVFGFAAVV	HLA-DRB1*09:01	214.00	1.3741	Non- allergen
	HLA-DRB1*01:01	162.00		
EFKISVFGFAAVVPA	HLA-DRB1*01:01	112.00	1.5196	Non- allergen
FKISVFGFAAVVPAQ	HLA-DRB1*01:01	138.00	1.3738	Non- allergen
FTLHHFAAPAPPAKL	HLA-DRB1*04:04	87.00	0.6040	Non- allergen
	HLA-DRB1*09:01	248.00		
	HLA-DRB5*01:01	151.00		
	HLA-DRB1*01:01	86.00		
KEEDAFNISAFGFVA	HLA-DRB1*09:01	172.00	0.7168	Non- allergen
KISVFGFAAVVPAQS	HLA-DRB1*08:02	118.00	0.8556	Non- allergen
	HLA-DRB1*09:01	45.00		
	HLA-DRB1*04:04	46.00		
	HLA-DRB1*04:05	134.00		
	HLA-DRB1*04:01	183.00		
	HLA-DRB1*01:01	32.00		
	HLA-DRB5*01:01	108.00		
	HLA-DRB1*07:01	218.00		
QAYYQGWIKFVKAAD	HLA-DRB1*04:05	142.00	0.8529	Non- allergen
SFTLHHFAAPAPPAK	HLA-DRB1*04:04	86.00	0.6518	Non- allergen
	HLA-DRB5*01:01	165.00		
	HLA-DRB1*01:01	92.00		
TLHHFAAPAPPAKLF	HLA-DRB5*01:01	147.00	0.5433	Non- allergen
	HLA-DRB1*04:04	229.00		
	HLA-DRB1*01:01	95.00		

VHQFTSPAPAAKVFL	HLA-DRB5*01:01	28.00	0.7514	Non- allergen
	HLA-DRB1*01:01	104.00		
VKVHQFTSPAPAAKV	HLA-DRB5*01:01	31.00	0.9198	Non- allergen
	HLA-DRB1*09:01	225.00		
	HLA-DRB1*04:04			
	HLA-DRB1*01:01	153.00		
		106.00		
YYQGWIKFVKAADEM	HLA-DRB1*04:04	60.00	0.5970	Non- allergen
	HLA-DRB1*04:05	116.00		

3.4.4. Tertiary modelling of $CD8^+$ and $CD4^+$ T-cell epitopes

The final predicted T-cell epitopes were subjected to docking simulation with chicken MHC alleles using AttractPep and locally installed packages on CHPC to evaluate the binding interaction between the alleles and the obtained epitopes. The chicken MHC alleles used for docking the CTL and HTL epitopes were BF2*21:01 (pdb: 4DOC) and pBL2*019:01 (pdb: 6KVM) , respectively. The results obtained after docking of CTL epitopes showed that epitopes FKISVFGFA, FNISAFGFV, FTSPAPAAK, KISVFGFAA and AQEAAAAAA bound with binding grooves of BF2*21:01 with binding energy of -142.867 kcal/mol, -137.498 kcal/mol, -99.143 kcal/mol, -114.176 kcal/mol and -93.170 kcal/ mol, respectively (Figure 3.1a). For the docking simulation of $CD4^+$ T-cell epitopes, it was found that epitopes EEEEFKISVFGFAAV, EEEFKISVFGFAAVV, EFKISVFGFAAVVPA, FKISVFGFAAVVPAQ and KEEDAFNISAFGFVA bound with binding grooves of HLA-DRB*01:01 with binding affinities of -133.454 kcal/mol, -118.721 kcal/mol, -135.814 kcal/mol, -137.521 kcal/mol and -123.062 kcal/mol, respectively (Figure 3.1b).



a) b)

Figure 3.1: Docking structures showing binding interaction of epitopes with HLA alleles: (a) CD8⁺ T cell epitopes binding to HLA-A*2:06. (b) CD4⁺ T-cell epitopes binding to HLA-DRB1*01:01.

3.4.5. Epitope merging for generation of multi-epitope subunit vaccine

The multiepitope vaccine candidate against *Eimeria* was constructed by joining together the prioritized final predicted 7 CTL epitopes and 21 HTL epitopes using the AAY and GPGPG flexible linkers. The CBT adjuvant (accession no. ABV74245.1) was attached at the N-terminal of the vaccine construct by EAAAK linker, to enhance the immunogenicity of the construct. This resulted in the multi-epitope vaccine containing 610 amino acid residues, which was further validated for antigenicity, allergenicity and physicochemical properties.

3.4.6. Antigenicity, Allergenicity, Solubility, and Physicochemical properties assessment

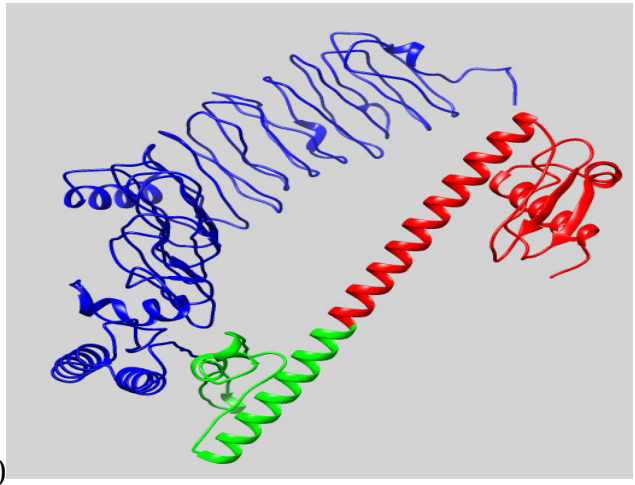
The constructed multiepitope vaccine was further subjected to various tools to validate its effectiveness as a vaccine. The vaccine was found to be antigenic with Vaxijen score of 0.5989, non-allergic and immunogenic (score= 10.152), making it a potentially good candidate to provoke an effective immune response in the host (Calis et al., 2013). The physiochemical properties evaluated using ProtParam server revealed that the designed multi-epitope vaccine had a molecular weight of 60.87 kDa, theoretical pI of 6.33 and instability index of 31.70. The obtained theoretical pI and instability index classified the vaccine protein as slightly acidic and stable. The half-life of the vaccine was assessed to be 30 hours (h) (*in vitro*) in mammalian reticulocytes was 30h; and > 20 and 10h in yeast and *E. coli*, *in vivo*. The aliphatic index and grand average of hydropathicity (GRAVY) were 66.39 and 0.209, respectively (Pandey et al.,

2018). The obtained aliphatic index and GRAVY suggests the designed vaccine is thermostable and hydrophobic in nature. The overall physiochemical properties and allergenicity results revealed that the multiepitope vaccine is immunogenic, non-allergenic and thermostable, making it appropriate for vaccine production (Dar et al., 2019).

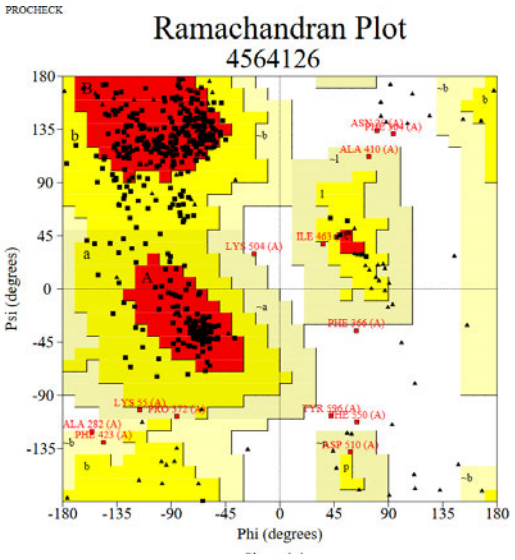
3.4.7. Tertiary structure prediction, refinement, and validation

The tertiary structure of the designed multi-epitope vaccine was predicted using the RaptorX server. Since the designed vaccine had no structural template, the RaptorX server was the suitable platform to generate the vaccine's tertiary structure. The server produced five different potential models which were validated using the PROCHECKER server. Model 4 was chosen from the models produced as potential vaccine structure with Ramachandran plot percent of 79.8% of residues in the favoured region. However, the validation showed that the designed vaccine peptide had some missing residues. Hence, the selected model was refined using GalaxyRefine server. The server also produced five potential vaccine models with an improved number of residues allowed in the favoured region (Table S3.1). The best model selected was Model 5 (Figure 3.2a), which showed an improved percentage of 80.9% in the Ramachandran plot analysis when revalidated after refinement (Figure 3.2b). Other favourable parameters obtained for the refined model included: GDT score of 0.8684, RMSD value of 0.626, MolProbability of 3.399, clash score of 84.1, and poor rotamers of 3.8. The selected model was further validated using ProSA- web, where a Z-score of -6.74. The Z-score obtained suggests that the designed vaccine is good quality since it lies within the vicinity of PBD X-ray experimental structures (Figure 3.2c).

(a)



b)



c)

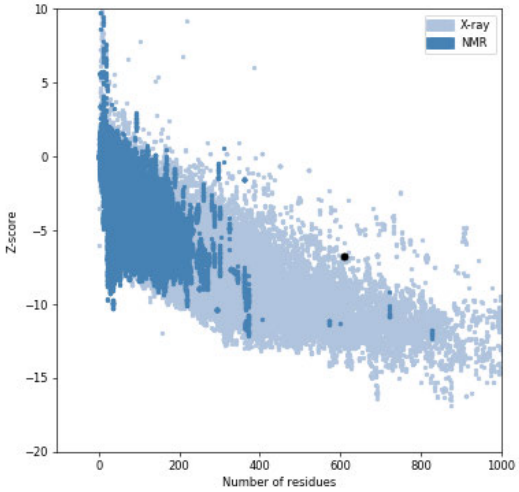


Figure 3.2: Vaccine construct structural validation analysis; a) Refined model of multiepitope vaccine construct (CD8$^+$ T-cell epitopes- green, CD4$^+$ T- cell epitopes- Blue and adjuvant- Red); (b) Ramachandran plot of refined vaccine (80.9% of the residues of the vaccine are present in the favoured region; c) PROSA web score plot indicating a Z-score= - 6.74, indicating overall model quality.

3.4.8. *Molecular docking of vaccine constructs with Toll-like receptor 5.*

The docking of the vaccine with receptor TLR5 complex produced 50 docked complexes, where the five best-docked complexes with lowest binding affinities ranging from -119.989 kcal/mol to -159.964 kcal/mol were selected. All the produced models were visualized

using the VMD server and Chimera v2.0 to observe the vaccine's interaction and binding to the TLR5 complex. The best-docked complex was selected as Model 47 with a binding affinity of -159.964 kcal/mol (Figure 3.3).

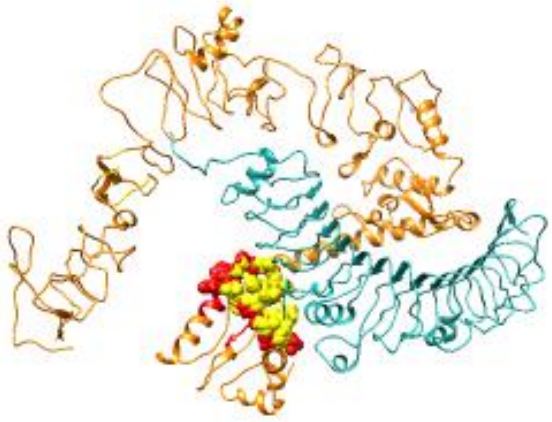
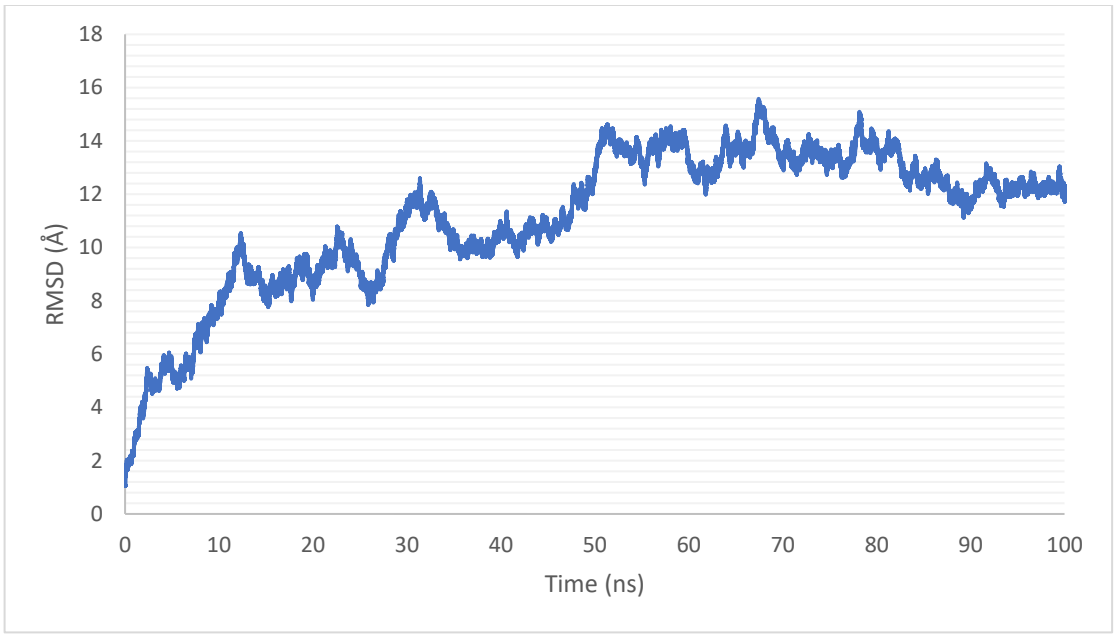


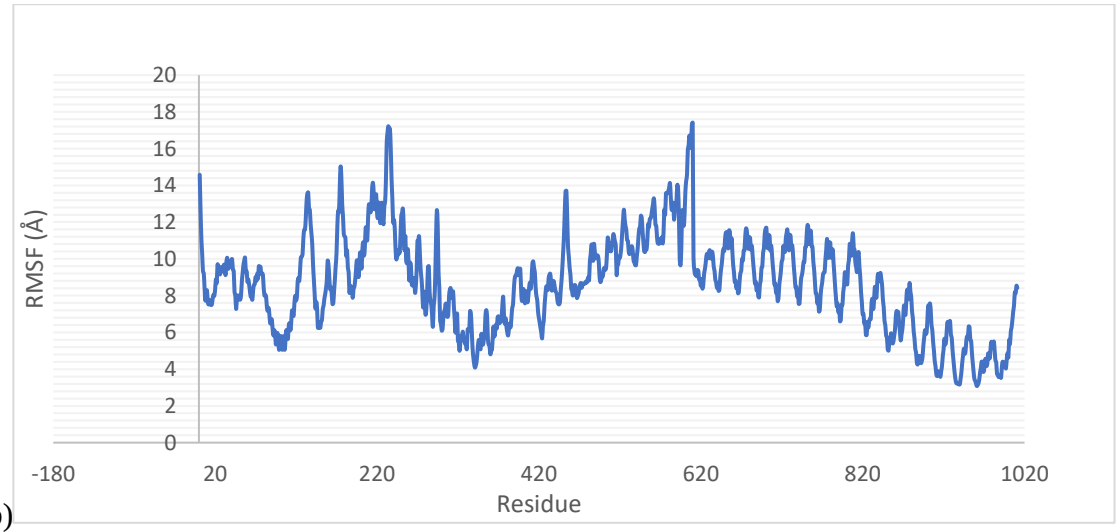
Figure 3.3: Molecular interaction 3D structure of the designed multiepitope vaccine(orange) with TLR5(blue) after docking analysis. Interacting residues indicated by red(TLR5) and yellow(vaccine).

3.4.9. Molecular dynamic simulation of docked vaccine complex

Molecular dynamics simulation was performed to assess the stability and binding of docked complex using parameters RMSD and RMSF. The structural flexibility was demonstrated by RMSD results (Figure 3.4 a), where fluctuation for backbone atoms of docked complex started at 1.5Å to 12Å. The complex reached a stable position with minimal fluctuation at 50 ns. RMSF analysis (Figure 3.4 b) revealed side chain atoms of docked complex that exhibited high interaction between vaccine and TLR5, with fluctuation regions at 110-230 and 510-650 residues.



(a)



(b)

Figure 3.4: Molecular dynamics simulation of docked complex at 100 ns time duration (a) RMSD plot showing structural stability of the docked vaccine-TLR5 complex through analysis of amino acid backbone; and (b) RMSF plot showing interaction of side chain atoms between vaccine construct and TLR5.

3.4.10. In silico codon optimization, cloning and expression of vaccine construct

To optimise the use of codons of the vaccine structure for maximal expression in *E. coli* (strain K12), the Java Codon Adaptation Tool (JCat) was used. The improved nucleotide sequence of vaccine was found to have CAI value of 1.0 and GC-content of 55.19. showing high probability

of expression of the vaccine in the *E. coli* K12 strain. Finally, *in silico* cloning of the improved sequence was performed using SnapGene software, where XhoI and BamHI restriction sites were added into the final vaccine and closed into the expression vector petDEST42 (7630 bp construct) (Figure 3.5).

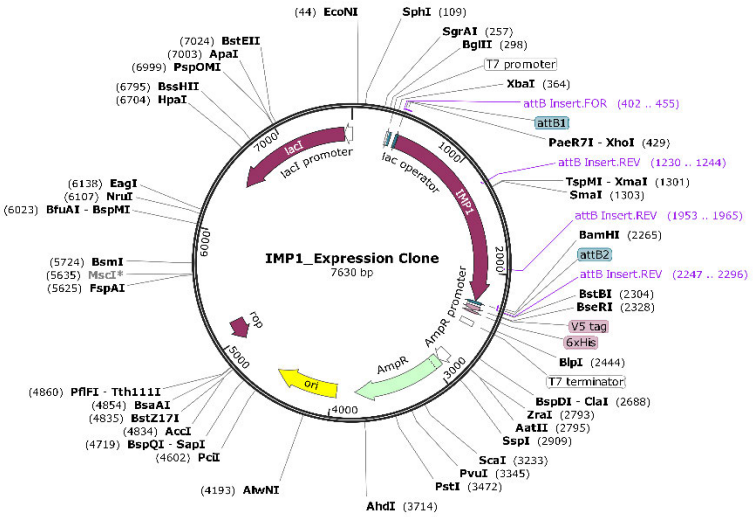
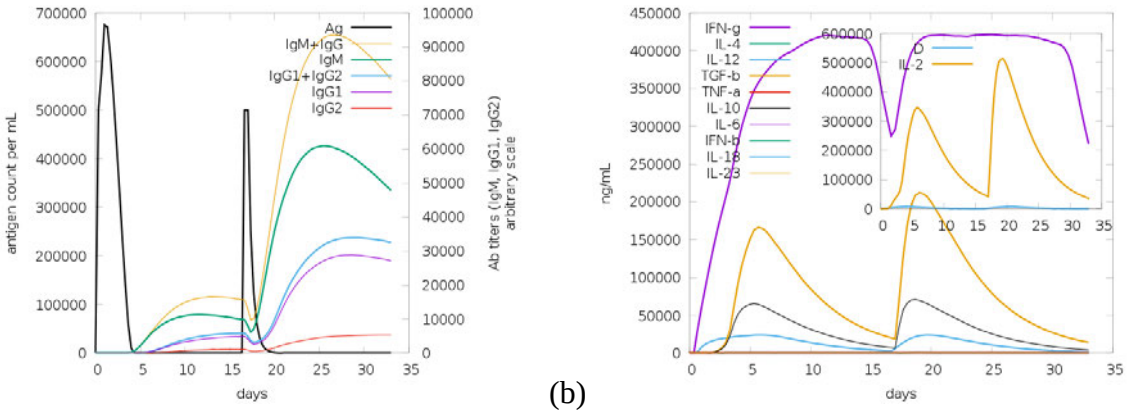


Figure 3.5: *In silico* cloning map for improved multiepitope sequence into petDEST42 vector, showing multiepitope vaccine labelled as IMP1 connected with XhoI (429) at C terminal and BamHI (2265) N terminal.

3.4.11. Immune simulations for vaccine construct

The results of immune simulation revealed different immune profiles produced by the vaccine, where the vaccine was observed to induce an immune response by the increase of antibodies upon administration to the simulation. The administration of the vaccine candidate showed a significant increase in tertiary immune response exhibited by high levels of IgG1 + IgG2, IgG + IgM, IgG1 and IgG2 compared to primary response represented by IgM (Figure 3.6a). Exposure of the B-cell population to the vaccine showed constant increased levels, developing memory cells to keep the vaccine's memory if the host encounters reinfection (Figure S3.2). The subsequent injection of vaccine construct also exhibited a significant increase in cytokines such as IFN-gamma, TGF-b, IL-10, IL-23, and IL-12 indicating good immune response (Figure 3.6b), correlating with the prediction of IFN-gamma epitopes in the vaccine. The decline in antigen level with each injection confirmed the presence of antibodies that effectively maintained the possibility of an antigenic surge.



(a) (b)

Fig 3.6: Immune simulation showing immune response through (a) production of Immunoglobulin in response to antigen injections (black vertical lines); and (b) Production of cytokines induced by subsequent injection of vaccine over 4 weeks.

3.5. DISCUSSION

Coccidiosis is a ubiquitous disease caused by *Eimeria* in livestock (Jenkins, 1998; Soutter et al., 2020; Turk, 1982). This disease has caused substantial economic loss globally in the chicken industry due to the high mortality of chickens leading to reduced productivity. Vaccination of poultry at an early age is crucial to curb the economic impact that coccidiosis currently pose to the poultry industry. Due to their rapid life cycle, consisting of multiple stages within a host, the complexity of *Eimeria* infections requires novel approaches in vaccine design to improve their efficacy. This study aimed to apply immunoinformatic and reverse vaccinology to generate a multi-epitope vaccine candidate capable of inducing targeted response against *Eimeria*. The immunoinformatic approach enabled the selection of specific immunogenic machinery of the parasite responsible for the disease rather than the inclusion of the whole cell, preventing undesirable immune response which may result in enhanced allergenicity of the parasite towards a host, reducing the time required for the design of the vaccine (Dar et al., 2019; Jacob et al., 1985; Pandey et al., 2018).

In this study, the genomic sequence of *Eimeria* IMP-1 antigen was exploited to predict and design an immunogenic multi-epitope vaccine candidate . The inclusion of different multiple epitopes in vaccine design may potentially provide maximum protection against various *Eimeria* spp. strains. Song et al. (2015) suggested that merging T-cell epitopes from different stages of the *Eimeria* life cycle could overcome the parasite's antigen complexity, which this

study aimed to explore through designing a multiepitope vaccine. To achieve this, the conserved sequences of IMP-1 antigen were selected and used to generate antigenic and immunogenic T-cell epitope candidates crucial for multiepitope vaccine construct. The use of T-cells capable of eliciting strong humoral and innate response in the vaccine construct ensured that the vaccine designed would confer double protection against the parasite (Ojha et al., 2020). The presence of $CD4^+$ helper T cells capable of stimulating IFN-γ and IL-4 cytokines in the construct further enhanced the potential of vaccine to activate an immune- stimulatory response through production of cytokines (danger signalling cells).

Tang et al. (2018) reported that IFN-γ and IL2 could elicit lymphocytes and are involved in Th1 mediated immune response and Th2 immune response, respectively. Hence the inclusion of epitopes capable of inducing cytokines was crucial for improving the efficacy of the vaccine. The final predicted T-cells (7 CTL epitopes and 21 HTL epitopes) were joined together by flexible linkers to form the vaccine construct with 610 aa. An ideal vaccine also depends on the effectiveness of adjuvant selected to improve the host immune response. In the current study, CTB adjuvant was added to the N-terminal of the vaccine peptide by EAAAK linker. Toxin derived adjuvant (such as CTB) has been used in several studies, where it was noted effective in enhancing the immunogenicity of multiepitope vaccines by inducing production of immunoglobulins (IgG and IgA) (Banerjee et al., 2020; Gupta and Kumar, 2020; Nosrati et al., 2019; Patronov and Doytchinova, 2013; Singh et al., 2020). The constructed vaccine was found to be immunogenic with a score of 10.152, antigenic with VaxiJen score of 0.5989 and non-allergenic, making it a good candidate for vaccine development.

The constructed vaccine consists of 610 amino acid residues with a molecular weight of 60.8 kDa with instability index of 31.70, fitting the criteria that vaccine proteins with molecular weight <110 kDa and instability index < 40 are relatively stable/good vaccine candidates (Dar et al., 2019; Singh et al., 2020). The evaluation of vaccine physiochemical properties further revealed the designed vaccine construct to be thermostable, acidic with highly hydrophobic nature (GRAVY= 0.209); physiochemically fitting for production. Similar findings were observed by Ojha et al. (2020). The designed vaccine was subjected to structural validation using a Ramachandran plot and ProSA with scores of 80.9% and -6.74, respectively. These scores indicated that the vaccine structure was of good quality, as the model lied in the vicinity of X-ray resolved structures in PDB. The vaccine was docked to the flagellin TLR5 receptor, an effective inducer of innate immune response (Atapour et al., 2019). Guptar et al. (2014)

reviewed the effectiveness of flagellin as an adjuvant according to published studies. They revealed flagellin to induce immune system in chickens through the production of cytokines by innate immune cells, confirming its effectiveness as TLR5 ligand.

Molecular docking of the vaccine candidate allowed analysis of binding interaction of vaccine to the receptor. The highest binding energy observed from the docking simulation revealed that the vaccine complex has a significant affinity to the receptor and can stimulate TLRs within the host leading to an improved immune response against *Eimeria*. Similar findings were obtained by Yin et al. (2013) when they evaluated *E. tenella* IMP1 and flagellin (TLR5 agonist) as potential *Eimeria* vaccine candidate. They proved through vaccination of three-week-old AA broiler chickens that recombinant EtIMP1-flagellin fusion protein enhanced immune response of chickens, making it an effective immunogen (Yin et al., 2013). Molecular docking results were further validated using molecular dynamics simulation (Figure 3.4), where obtained RMSD and RMSF revealed a stable and flexible docked complex, exhibiting continuing interaction of the vaccine construct and the TLR5 with minimal fluctuations (Khatoon et al., 2018; Ojha et al., 2020; Yadav et al., 2020).

Zhang et al. (2020) reported upregulation of TL5 in *E. tenella* infection, which triggered activation of pro-inflammatory cytokines IL-2 and IFN- γ. This support immune simulation findings obtain in this study, where administration of vaccine construct induced increased production of IFN- γ and IL genes (Figure 3.6b). Similar findings were reported by Liu et al. (2018) in a separate study, evaluating efficacy of DNA vaccine (pVAX-Ea14–3-3) against *Eimeria* infection. The authors confirmed production IFN- γ, IL-2 and IL-4 in high levels provided effective protection against *Eimeria* infection (*E. acervulina, E. maxima and E. tenella,*). High levels of cytokines in host, activate microphages that inhibit and kill *Eimeria*. As observed in this study (figure S3.1d), subsequent doses of vaccine activated growth of microphages, which remained elevated throughout the simulation. The production of IgA upon initial administration of vaccine into the simulation stimulated host immune response through the production of high levels of antibodies that aid in primary response (IgM) and tertiary response (i.e., IgG1 + IgG2, IgG1 and IgG2).

The gradual increase of B-cell memory cells and antibodies after subsequent exposure to three vaccine injections confirmed the vaccine's effectiveness to host when exposed for a duration of time, consistent with vaccine’s immunogenicity. The increased, continuous production of T-cells (Figure S3.1c) and cytokines such as interferon (IFN)-γ (Figure 3.4b) show the

vaccine's potential to exert a protective effect against parasite infections, since they are crucial for cellular immune response and anticoccidial immunity towards coccidiosis (Onile et al., 2020; Tang et al., 2019). Previous literature suggests that *in silico* immune simulations can be consistent with real immune response exhibited by affected host against pathogens (Rapin et al., 2010). Hence, it can be assumed that simulation outcome for the present study may provide a realistic insight on efficiency of the designed vaccine against *Eimeria* through induction of protective immune response.

3.6. CONCLUSION

From the present study, the design of a multi-epitope vaccine was achieved successfully using the immunoinformatic approach. The vaccine designed exhibited all the parameters crucial for potential vaccine candidates, as it effectively induced immune response through production of cytokines in an immune simulation technique. Based on this study, it might be promising to focus on specific regions of the parasite's protein rather than large protein residues as this might contribute to the reduction of parasite's antigen complexity. Also, the combination of multiple T-cells from different phases of the *Eimeria* life cycle may effectively confer ideal protection against multiple *Eimeria* species though this still requires further experimental validation(s). This would also minimise any possible negative effects of using the whole genome of the parasite, lowing risk of reinfection. In the present study, a multiepitope vaccine candidate containing 7 CTL epitopes and 21 HTL epitopes and an adjuvant resulted in a vaccine construct that significantly enhanced immune protection of the host by prediction. It can be concluded that the immunoinformatic approaches explored in the prediction of the designed vaccine candidate yielded promising results. It is highly recommended that further studies and experimental validation be done on the results obtained and reported in the current study for confirmation purposes and to validate the safety and efficacy of the vaccine.

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CHAPTER FOUR

Designing T- cell epitope-based vaccine against *Eimeria* from Microneme Protein 2 (MIC2) antigen using immunoinformatic approach

4.1. ABSTRACT

Avian coccidiosis is a parasitic disease globally recognised for incurring significant production loss to the poultry industry. It is a consequence of single or multiple *Eimeria* spp. infection, characterized by malabsorption, enteritis, and poor productivity in birds. The currently used preventive measures to control coccidiosis are dependent on chemoprophylaxis therapy and live anticoccidial vaccines, which has led to drug resistance in parasites and the presence of drug residues in food, compromising food security. The industry's economic loss has led to an imperative search for novel vaccines or drugs that can induce protection against multiple *Eimeria* species to control coccidiosis. This study explored Microneme Protein-2 (MIC2) antigen to predict and develop an epitope-based vaccine against coccidiosis by identifying antigenic T-cell epitopes using immunoinformatic techniques. A total of seven (7) $CD8^+$ and 12 $CD4^+$ T-cell epitopes were identified and merged together along with an adjuvant (Monophosphoryl Lipid A) using EAAK, AAY and GPGPG linkers to produce a vaccine multiepitope candidate. The designed multi-epitope vaccine (molecular weight of 54556.66 Da) was evaluated for physiochemical parameters, refined and validated through Ramanchandran plot analysis. Molecular docking of vaccine with Toll-like receptor 4 (TLR4) was performed to determine interaction and stability of vaccine construct**.** Physiochemical parameters of vaccine projected it as thermostable (instability index of 25.18), hydrophilic, induce immunity through production of antibodies and cytokines, which may be vital in hindering the surface entry of parasite into host while boosting the immune system host, preventing infection. These findings are crucial information for experimental design of cost-efficient novel vaccine against *Eimeria.*

Keywords: Coccidiosis, *Eimeria*, Vaccine, MIC2, Immunoinformatic, Immune response

4.2. INTRODUCTION

Chicken coccidiosis is a consequence of single or multiple *Eimeria* spp. infection. This disease is recognised for incurring significant production loss to the poultry industry worldwide (Zhang et al., 2020; Song et al., 2015). There are seven different *Eimeria* species reported to cause an impact on chickens, with *E. tenella* being most prevalent and pathogenic. *Eimeria* infections are characterized by malabsorption, enteritis, and poor feed conversion, drastically reducing productivity and health of birds (Quiroz -Castañeda and Dantán-González, 2015). Poultry farmers are mainly dependent on chemoprophylaxis therapy and live anticoccidial vaccines to treat and control coccidiosis. These have proved effective but with noted constraints such as the presence of drug-resistant strains due to continued use of anticoccidial and high cost of vaccine production. This had led to increased efforts in the search for novel preventive strategies against *Eimeria*, targeting various developmental stages of the parasite's life cycle, hindering parasite invasion while exhibiting limited setbacks.

Recent studies have demonstrated promising alternate vaccines that include antigens isolated from *Eimeria* spp. as potential vaccine candidates and effective in curbing coccidiosis (Patronov and Doytchinova, 2013). Research has shown that using these immunogenic and highly conserved antigens as part of the vaccine stimulates the host's enhanced immune response. Micronemes are membrane-bound organelles situated beneath the cell membrane of the apical complex(Banerjee et al., 2020). They aid in the secretion of proteins vital for parasite and host interaction such as host invasion, recognition, and motility(Aziz et al., 2019; Valdivia-Olarte et al., 2015). *Eimeria* microneme protein-2 (MIC2) is an acidic protein that was first discovered in the cell membrane of *E. tenella* sporozoites by Tomley et al. (1996).

Previously, the antigen was characterized through several studies as highly conserved and immunogenic amongst *E. tenella* strains (Atapour et al., 2019; Saha and Raghava, 2006). It was reported to be expressed in all crucial developmental phases of *E. tenella* and interacts with the host cell, serving as a good potential candidate for vaccine development. These proteins are located in the transmembrane, where they play a vital role in hindering parasite's invasion into intestinal epithelial cells to evade coccidiosis (Zhao et al., 2019). Yan et al. (2018) characterized and investigated protective efficacy of *E. tenella* Micromeme2 (EtMIC2), where it was found to significantly increase body weight gain, reduced lesion score and oocyst shedding in chickens. It was further observed to play a crucial role in parasite cell invasion by

obstructing *E. tenella* sporozoites from entering host cells, confirming its role as a promising vaccine candidate.

Dalloul et al. (2005) affirmed administration in ovo of CpG ODNs and an *Eimeria* recombinant microneme protein (MIC2), which exhibited enhanced immunity and adjuvant effects; with improved weight gain following *Eimeria* infections. Shi et al. (2014) evaluated the protective efficacies of a DNA vaccine consisting of EtMic2 protein and chicken interleukin-18 for DNA coccidiosis, and it was found that immunization of chicken with recombinant protein improved immune protection against challenge. Similar results were obtained by Huang et al. (2015) when evaluating recombinant protein and DNA vaccine encoding EmMIC2 against *E. maxima* challenge, where they found the treatment to reduce jejunum lesions, bodyweight loss, decline in oocyst shedding and decreased ratio.

Following the various successes reported on MIC2, this study explored *Eimeria* microneme protein-2 antigen with the aim to design an multiepitope-based vaccine against *Eimeria* by identifying antigenic T-cell epitopes using immunoinformatic techniques

4.3. **METHODS AND MATERIALS**

4.3.1. *Protein sequences availability and identification of conserved sequences*

Eimeria microneme protein-2 (MIC2) protein sequences were obtained from the National Centre for Biotechnology Information- NCBI[1]. The sequences were then aligned using multiple sequence alignment (MSA) to generate preserved with minimum of 15 amino acid residues. The MSA was performed using default parameters from online server, CLUSTALW[2](Adhikari and Rahman, 2017; Kamthania et al., 2019; Mellet et al., 2019; Thompson et al., 1994).

4.3.2. Transmembrane structural analysis and antigenicity of conserved regions

In this study, the selected conserved regions were exposed to antigenicity testing using the VaxiJen v2.0 Server[3], with a threshold set to be 0.4. The sequences that were identified as probable antigens were selected and tested for transmembrane helix properties in the TMHMM v2.0 server[4]. The TMHMM server was used to identify outer membrane protein sequences from those previously selected as conserved.

4.3.3. T-cell epitopes Prediction

Human HLA allele selection

Since there is no data currently available in the immunoinformatic software for chicken B-F alleles that can be used for MHC-epitope binding prediction, MHC class-I alleles (HLA-A and -DRB in humans) were chosen for this study to substitute the chicken MHC alleles (Magdeldin et al., 2012). The substitution of chicken MHC allele with human alleles is supported by previously reported similarities observed between MHC Class I and B-F alleles (Osman et al., 2016; Tan et al., 2016).

4.3.3.1. $CD8^+$ T-cell epitope prediction.

The outer membrane protein sequences identified from the selected conserved regions were submitted to NetCTL v1.2 tool, to generate potential nonamers that can activate $CD8^+$ T cells through interacting with major histocompatibility complex (MHC) class I (HLA alleles)The resulting nonamers were subjected to the IEDB analysis tool[5] to predict epitopes (Zhang et al., 2020). The nonamers were analysed using the Stabilized Matrix Base Method (SMM) (Nielsen et al., 2007). The parameters for identifying MHC-I binding alleles selected included the amino acid length of peptide set to 9.0, the IC_{50} value< 250, and the MHC source species selected as human. The obtained epitopes were examined for antigenicity, with a threshold set as 0.5 as the main parameter. Sequences that were detected to be above the set threshold were selected as probable antigens. The $CD8^+$ T-cell epitopes were further tested for immunogenicity using the MHC I IEDB immunogenicity tool[6] (Higgs et al., 2006).

The generated antigenic and immunogenic epitopes were tested for conservancy using EIDB online server[10] (Bui et al., 2007) and allergenicity using AllerTop v2.0 tool to filter out allergenic sequences (Dimitrov et al., 2013).

4.3.3.2. $CD4^+$ T-cell epitope prediction.

IEDB MHC II binding tool[7] was used to predict HTL epitopes and their MHC class II alleles (Higgs et al., 2006; Keestra and van Putten, 2008). The prediction method used was SMM-align (stabilization matrix alignment), allele length was set at 15, and a threshold for IC_{50} was < 250. The identified HTL epitopes were subjected to the IFNepitope tool[8] to predict epitopes that could induce cytokine IFN-γ. The IL-4 inducers were predicted using the IL4pred tool[9].The

resulting epitopes underwent further scrutiny using similar immunoinformatic tools as $CD8^+$ T-cell epitopes, where they were tested for antigenicity with a threshold set as 0.5, overlapping ability and allergenicity.

4.3.4. *Tertiary structure design of the predicted T-cell epitopes.*

The final predicted epitopes were docked with two chicken BF alleles to determine the best binding interaction between $CD8^+$, $CD4^+$ T-cell epitopes and the MHC-alleles. The chicken BF alleles BF2*2101 (pdb: 4D0C) and pBL2*019:01 (pdb: 6KVM) were obtained from the RCSB Protein Data Bank (PDB)[11]. The chicken alleles were first prepared using naccess[12]where the active and passive residues were generated and later viewed in chimera v1.14. Molecular docking of epitopes with PDB structure of the binding MHC alleles was performed using the ATTRACT Peptide online[13] to generate haddock files to be used to complete docking (de Vries et al., 2017; Pandey et al., 2018). The prepared MHC-allele structures with haddock files from AttractPep were further subjected to the CHPC platform to complete docking simulation and to obtain flexibility of the structure, using locally installed attract. A total of 50 models were generated for each epitope. The model with the lowest binding energy and which properly occupied the receptor was selected and then combined with B-F allele to form one epitope-complex. Structural illustrations of generated models were visualised using Chimera[14] (Pettersen et al., 2004).

4.3.5. Multi-epitope subunit vaccine construction

Construction of the multiepitope vaccine was achieved by the merging of the final best candidates of T cell epitopes using linkers and adjuvants. The $CD8^+$ T cell epitopes were joined by the AAY linkers and $CD4^+$ T cell epitopes by GPGPG linkers and the adjuvant was attached to the N-terminal of the vaccine with EAAK linker. These linkers provided extended flexibility to the peptides making up the vaccine for increased stability. The addition of the adjuvant to the vaccine is crucial as it enhances the immunogenicity of the vaccine (Dong et al., 2020). The adjuvant added to the vaccine construct was Monophosphoryl lipid A.

4.3.6. Antigenicity, Allergenicity, Solubility, and Physicochemical properties evaluation

The resulting multiepitope vaccine sequence was checked for antigenicity using Vaxijen v2.0 server[15]. The prediction of vaccine's antigenic nature ensured the vaccine's ability to bind and interact with the receptor during the docking stage (Doytchinova et al., 2007).

AllerTop v2.0 was used to determine further whether the constructed vaccine was an allergen or non-allergen. To assess the physicochemical properties of the vaccine, a FASTA sequence of the vaccine was subjected to ProtParam53 web server[16]from the Expert Protein Analysis System (EXPASY) to calculate the number of amino acids of the vaccine, molecular weight (kDa), theoretical isoelectric point (pI), estimated half-life, instability index, aliphatic index, hydropathicity GRAVY.

4.3.7. 3D modelling, refinement, and validation of vaccine construct

The 3D structure of the designed multiepitope vaccine protein was predicted and generated using trRosetta[2222]. Molecular refinement of the vaccine tertiary structure was performed using the GalaxyRefine server[18] (Banerjee et al., 2020; Gupta and Kumar, 2020; Nosrati et al., 2019; Patronov and Doytchinova, 2013; Singh et al., 2020). Refinement of the tertiary structure was performed to enhance the structural quality of the vaccine protein. Five refined models of the vaccine constructs were predicted using the GalaxyRefine server from structural perturbations and structural relaxations (Ko et al., 2012).

The overall quality of the selected tertiary model for the designed vaccine was determined using the Ramachandran plot, generated from online server PROCHECK. This server focuses on analysing residue-by-residue and overall structure geometry of a protein structure to obtain its stereochemical quality. ProSA-web[19] was also employed for final structure validation by generating a Z-score for confirmation (Laskowski et al., 1993).

4.3.8. Molecular dynamics simulation of the vaccine construct

Energy minimization of vaccine construct and TLR5 complex was achieved by molecular dynamics simulation technique, using an AMBER 14 package (literature et al., 2014). The input proteins were described using FF14SB (Maier et al., 2015) and topologies of the vaccine structure were generated using the LEAP module of AMBER 14. This was done by introduction of ions (protons and Cl-) into the orthorhombic solvation box filled with water molecules, to neutralize the system. The energy minimization of the protein was done to obtain the lowest energy of the protein (Ojha et al., 2020). This step was carried out for 10000 steps, where the steepest descents were reached at 500 steps with 9500 conjugate gradients, followed by full minimization of 2000 steps. The density of the water was controlled with 4 ns while equilibration was 2 ns at 300 K. The molecular dynamic simulation was run for 100 ns of NVT (constant number N, volume V and temperature, where simulations were ran at different initial

speeds. After simulation, parameters Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuations (RMSF) were analysed, and results were graphed.

4.3.9. In silico codon optimization, cloning and expression of vaccine construct

Codon optimization of multiepitope construct sequence was performed using online server Java Codon Adaptation Tool (JCat)[20] to evaluate immuno-reactivity of vaccine and to obtain improved nucleotide sequence with probability of high-level expression in a selected expression vector. The tool is based on codon adaptation index (CAI) and percentage GC content, with optimal CAI score of optimized gene sequence (0.8-1.0) and GC% (30-70%) (Dar et al., 2019; Yadav et al., 2020). To clone and express the optimized nucleotide sequence of vaccine in *E. coli* (strain K12) host, XhoI (CTCGAG) and BamHI (GGATCC) restriction sites were introduced to the C- and N-terminal sites of the final vaccine construct, respectively. In silico cloning vaccine was performed using SnapGene software, where the improved sequence consisting of restriction sites was inserted into suitable expression vector, to evaluate the expression.

4.3.10. *Vaccine candidate in silico immune simulations*

Immune simulations of vaccine peptide were performed using the C-ImmSim server[21], to depict and confirm vaccine's immunogenicity . All simulation parameters used for simulation were set at default (Shey et al., 2019).

4.4. RESULTS

4.4.1. *Eimeria MIC2 protein sequences availability and identification of conserved sequences*

A total of 19 sequences for *Eimeria* MIC2 antigen were retrieved from NCBI. The genome's conserved sequences were created and selected using the CLUSTALW online server through multiple sequence alignment, where about 33 conserved sequences were selected.

4.4.2. Transmembrane structural analysis and antigenicity of conserved regions.

The obtained preserved sequences were tested for antigenicity with default parameters of threshold value set at ≥0.4, in Vaxijen v2.0 server. It was found that all the sequences fulfilled the antigenicity property of set threshold with Vaxijen score ranging from 0.4191 to 0.9825 (Table 4.1). These sequences were then used for further prediction analysis. The transmembrane analysis detected a total of 21 conserved sequences that fulfilled criteria of the exomembrane properties (Table 4.1).

Table 4.1: Conserved sequences of genome sequence of *Eimeria*.

Antigenicity Score	Conserved sequences	Trans-membrane helix
0.5155	MARALSLVALGLLFSLPPSSAVRTKVPGGDSFSPDSGVLDGADA	outside
0.5886	MARALSLVALGLLFSLPPSSAVRTRVPGEDSFSPESGVLSGTDA	outside
0.5914	MARTFSLIAFGLLFSLHSTSAIRTKVPGDDPTASDASLAVASST	outside
0.6814	MARALSLIALGLLFSLPSTSAVRTKVPGDEPSGPDSALTAGSPT	outside
0.5458	VAVVDESGSIGTTNYARVRSFLQGFAQTMPLSPDDVRVGLVTFGTKSVVRWELKDPTAQD	outside
0.7361	ALVVDESGSIGNTNYAKVRTFLGTFASSMPVSPEDVRIGLITFGTNAVTRWDLASPKAQN	outside
0.4527	VAVIDESGSIGTANFDKVRKFLSQFGSSLPLSSEDVRVGLVSFGTRAVKHWDLKDPRAQS	outside
0.5198	MLVVDESGSIGTSNFRKVRQFIEDFVNSMPISPEDVRVGLITFATRSKVRWNLSDPKATN	outside
0.8677	PEQRPTLPGLVEGNCGRLTVRSGLNVDESIKVTSAGWTKSERDFVVSLVAD	outside
0.6910	PERRPIVPGLVEGNCGRLTVRNGLSVDETIKVTSAGWTKSERDFIVSLVAD	outside
0.5796	GDRASLGGLLEANCERLTFRGGFKLGDTLKVTASGWGKSNADFLLELATD	outside
0.9526	RGNHGVGGFAEAHCNRLTVRGGLQEKEAVKVTANGWGKGDADFFVELVTD	outside
1.1614	EEAPKGEGGQEKPSVPLLAVRIRGSGDDKESSPQSAVLLYGSDESEPTEV	outside
1.1330	EEAPKGEGGQEKPSVPLIAVRIHGSGGDKGESAPQSAVLLYGNDESEPTEV	outside
0.9825	SKSEADGDLKEQPSIPVLGVRIPGSDSADGKARQPVVLVYGDGEEAPTEF	outside
0.7950	SKTDEGPVKEQPSIPIVGVRIPGSANENGESRKPAVLVYGEGESAPKEF	outside
0.4973	CADQAPGLIREEPCTMPACRADAHCGEFGAWSEWSATCGSATRYREREGYENPPASGGGL	outside
0.4840	CAEQPPGLIRMEPCTMPACKVDAYCGEFGPWSEWSTTCGSATRQRERQGYENPPASGGGL	outside
0.4623	CAEQSPGLIRTEPCSMPACRVDAHCGEFGPWSEWSTTCGSATRQRERQDYENPPASGGGL	outside
0.4790	CAEQPPGLIRTQPCTMPVCKTDAHCGEFGPWSEWSTTCGTATRKRQREGYNSPPAAGGGL	outside
0.4191	CAQQPPGLMRTEPCTMPACKIDAHCGDFGPWSEWSTTCGSATRQRVRQGYEDPPASGGGL	outside

4.4.3. T-cell epitopes Predictions.

4.4.3.1. $CD8^+$ T-cell epitopes prediction

The preserved sequences of MIC2 antigen were subjected to the NetCTL v1.2 server, where a total of 950 receptor-specific, immunogenic nonamers of $CD8^+$ T cell epitopes were found and subjected to the IEDB MHC-I prediction tool. Scrutinization of the nonamers based on SSM-based method and the IC_{50} value parameter < 250, detected 277 epitopes that interacted with one to eight MHC alleles. The identified epitopes were further tested for antigenicity with a threshold value set at ≥0.5 in the Vaxijen v2.0 server. A total of 95 $CD8^+$ T-cell epitopes were detected to be antigenic in nature, with Vaxijen score ranging from 0.5028 to 1.9864. The epitopes found to be antigenic were tested for immunogenicity to ensure their capability to induce immune response effectively. Only 58 sequences were identified to have a positive immunogenicity score. The obtained epitopes were then subjected to conservancy analysis, which detected 23 epitopes to be conserved. The conserved epitopes also underwent allergenicity analysis using AllerTop v2.0 server, where seven(7) $CD8^+$epitopes, AVRIHGSGG, EAHCNRLTV, GLITFGTNA, KPSVPLIAV, KVRTFLGTF, RTFLGTFAS and VSFGTRAVK were found to be non-allergens and were regarded as final predicted epitopes. The results for the identified final $CD8^+$ predicted epitopes, IC_{50}, antigenicity and allergenicity scores are presented in Table 4.2.

Table 4.2: Final predicted $CD8^+$ T cells epitopes, 100 overlapped with $CD4^+$ T cell epitopes and interacting with MHC 1 alleles

Epitopes	HLA alleles	IC_{50}	Antigenicity	Allergenicity
AVRIHGSGG	HLA-A*30:01	86.292	1.3876	Non allergen
EAHCNRLTV	HLA-A*68:02	102.70	1.0438	Non allergen
GLITFGTNA	HLA-A*02:03	34.352	1.4067	Non- allergen
KPSVPLIAV	HLA-B*07:02	76.893	0.5671	Non- allergen
KVRTFLGTF	HLA-A*30:01	104.705	0.9610	Non- allergen
	HLA-A*32:01	114.009		
RTFLGTFAS	HLA-A*32:01	81.272	1.2848	Non- allergen
VSFGTRAVK	HLA-A*11:01	41.908	0.8286	Non- allergen
	HLA-A*30:01	129.410		
	HLA-A*03:01	132.678		
	HLA-A*68:01	133.195		
	HLA-A*31:01	209.156		

4.4.3.2. $CD4^+$ T-cell (HTL) epitopes prediction

For the prediction of HTL epitopes, all 21 conserved sequences that fulfilled the transmembrane analysis were subjected to the IEDB MHC-II prediction module and VaxiJen server for the prediction of interaction with HLA alleles and antigenicity score, respectively. A total of 131 epitopes that fulfilled both the IEDB tool and Vaxijen parameters of IC_{50} value < 250 and ≥ 0.5 antigenicity score were obtained as potential HTL epitopes. The predicted $CD4^+$ T-cell epitopes were tested for conservancy, where 56 epitopes were selected as conserved. The conserved epitopes were then subjected to IFNepitope and IL-4pred immunoinformatic tools. Herein, a total of 27 $CD4^+$ T-cell epitopes that exhibited both IFN-gamma and IL-4 inducer properties were selected as potential $CD4^+$ T-cell epitopes. Prediction of these epitopes enhanced immunogenic capacity of the potential vaccine. These epitopes were subjected to AllerTop v2.0 for allergenicity analysis, where the analysis revealed only 12 epitopes to be non-allergenic. These epitopes were then selected as final suitable predicted HTL epitopes candidates for vaccine development since they do not cause any allergic reactions to the host (Table 4.3).

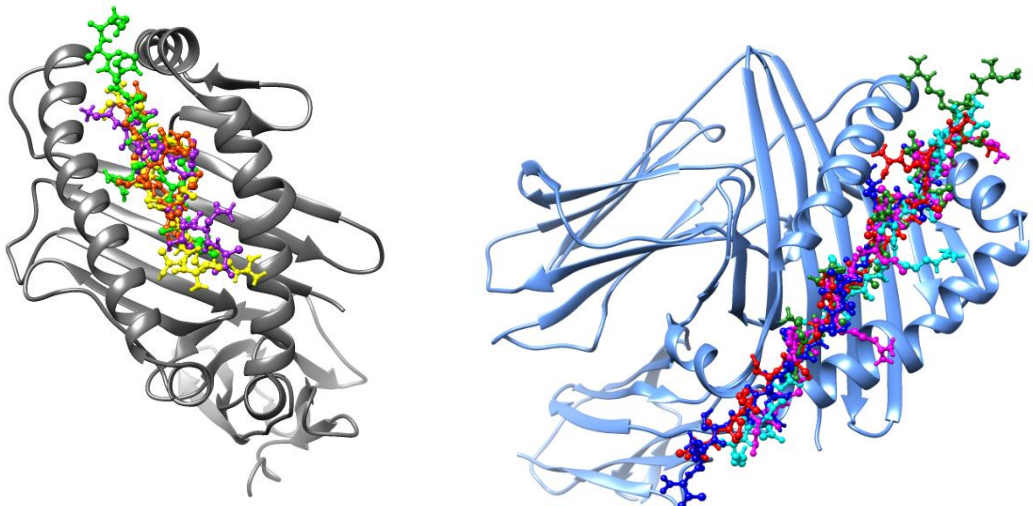
Table 4.3: Final predicted $CD4^+$ T cells epitopes, 100 overlapped with $CD8^+$ T cell epitopes and interacting with MHC 1 alleles

Epitope	HLA allele	IC_{50}	Antigenicity	Allergenicity
DVRVGLITFATRSKV	HLA-DRB5*01:01	102.00	1.0969	Non-allergen
	HLA-DRB1*11:01	113.00		
	HLA-DRB1*07:01	168.00		
	HLA-DRB1*01:01	145.00		
DVRVGLVSFGTRAVK	HLA-DRB1*15:01	122.00	1.0935	Non-allergen
	HLA-DRB1*04:01	163.00		
	HLA-DRB1*09:01	197.00		
	HLA-DRB1*07:01	149.00		
	HLA-DRB1*01:01	59.00		
EKPSVPLIAVRIHGS	HLA-DRB4*01:01	240.00	0.7644	Non-allergen
	HLA-DRB1*01:01	80.00		
KPSVPLIAVRIHGSG	HLA-DRB4*01:01	241.00	1.1992	Non-allergen
	HLA-DRB1*01:01	79.00		
KVRTFLGTFASSMPV	HLA-DRB1*04:01	108.00	1.1871	Non-allergen
	HLA-DRB1*01:01	18.00		
	HLA-DRB1*07:01	40.00		
	HLA-DRB1*04:05	120.00		
	HLA-DRB1*09:01	236.00		
	HLA-DRB1*04:04	155.00		
	HLA-DRB5*01:01	214.00		
LTVRGGLQEKEAVKV	HLA-DRB1*13:02	190.00	0.7403	Non-allergen
QEKPSVPLIAVRIHG	HLA-DRB1*01:01	147.00	0.8810	Non-allergen
RIGLITFGTNAVTRW	HLA-DRB1*04:01	148.00	0.5327	Non-allergen
	HLA-DRB1*15:01	144.00		
	HLA-DRB1*04:04	160.00		
	HLA-DRB1*01:01	68.00		
RVGLITFATRSKVRW	HLA-DRB1*11:01	97.00	0.6674	Non-allergen
	HLA-DRB5*01:01	94.00		

	HLA-DRB1*07:01	169.00		
	HLA-DRB1*01:01	202.00		
VGLITFATRSKVRWN	HLA-DRB1*11:01	99.00	0.5103	Non-allergen
	HLA-DRB5*01:01	98.00		
	HLA-DRB1*07:01	174.00		
VPLIAVRIHGSGGDK	HLA-DRB1*01:01	163.00	1.1406	Non-allergen
VRVGLVSFGTRAVKH	HLA-DRB1*04:01	168.00	0.8053	Non-allergen
	HLA-DRB1*15:01	142.00		
	HLA-DRB1*09:01	197.00		
	HLA-DRB1*07:01	146.00		
	HLA-DRB1*01:01	64.00		

4.4.4. Tertiary structure design of the predicted T-cell epitopes.

For the 3D modelling of obtained epitopes, the final predicted T-cell epitopes were subjected to docking simulation with chicken MHC alleles using AttractPep and CHPC to evaluate the binding interaction between the alleles and the obtained epitopes. The most conserved MHC-I and II HLA alleles detected for both epitopes were HLA-A*30:01 and HLA-DRB1*01:01, however, the MHC alleles used for docking the T-cell epitopes were chicken B-F alleles BF2*21:01 (pdb: 4DOC) and pBL2*019:01 (pdb: 6KVM), respectively. The choice of allele used for docking was mainly based on the antigens and protozoans' target organism. The results obtained after docking of $CD8^+$ epitopes showed that epitopes bound with binding grooves of BF2*21:01 with binding energy ranging from -100.488 kcal/mol to -156 kcal/mol. Binding affinities of epitopes was recorded as AVRIHGSGG (-127.178 kcal/mol, orange-red), EAHCNRLTV (-121.881 kcal/mol, green), GLITFGTNA (-100.488 kcal/mol, purple) and RTFLGTFAS (-156.594 kcal/mol, yellow) (Figure 4.1a). For the docking simulation of $CD4^+$ T-cell epitopes, it was found that epitopes DVRVGLITFATRSKV(blue), DVRVGLVSFGTRAVK (forest green), KPSVPLIAVRIHGSG (magenta), KVRTFLGTFASSMPV (red) and VPLIAVRIHGSGGDK (cyan) bound with binding grooves of pBL2*019:01 with binding affinities of -167.273 kcal/mol, -175.100 kcal/mol, -165.670 kcal/mol, -159.896 kcal/mol and -143.666 kcal/mol , respectively (Figure 4.1b).



a) b)

Figure 4.1: Docking structures showing binding interaction of epitopes with chicken MHC alleles (a) $CD8^+$ T cell epitopes binding to BF2*21:01. (b) $CD4^+$ T-cell epitopes binding to pBL2*019:01

4.4.5. Multi-epitope subunit vaccine construction

The shortlisted 7 $CD8^+$ T-cell epitopes, 12 HTL epitopes and monophosphoryl lipid A adjuvant with accession No. QFN32858.1 were linked together to form multiepitope vaccine construct using AAY and GPGPG flexible linkers. The adjuvant was attached at the N-terminal of the vaccine construct by EAAAK linker. This resulted in a multi-epitope vaccine candidate containing 512 amino acid residues, which was further evaluated for antigenicity, allergenicity and physiochemical properties.

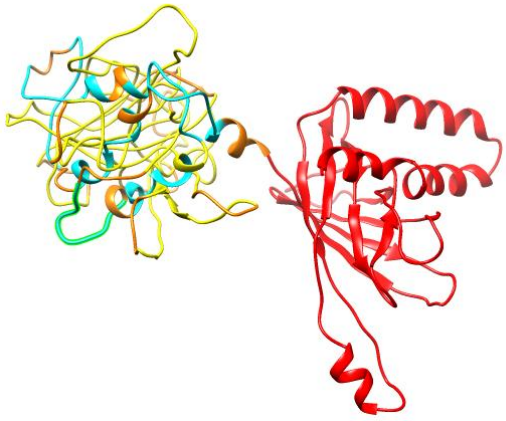
4.4.6. Antigenicity, allergenicity, solubility, and physiochemical properties evaluation

The designed vaccine construct was further subjected to a series of bioinformatics tools for validation purpose. The vaccine candidate was noted to be antigenic with VaxiJen score of 0.5103. The immunogenicity and allergenicity analyses showed the vaccine to be non-allergenic and immunogenic (score= 9.419). The physiochemical properties revealed the vaccine's molecular weight as 54556.66 Da, theoretical pI of 10.26, the instability index of 25.18 and aliphatic index of 76. 56, indicating the vaccine as slightly basic but stable to induce immunogenic reaction and to withstand multiple temperatures, which is crucial for an effective vaccine. The vaccine had an estimated half-life of 30 hours (h) in mammalian reticulocytes, *in vitro*; and > 20 and 10h in yeast and *E. coli*, *in vivo*. The vaccine was found to be hydrophilic

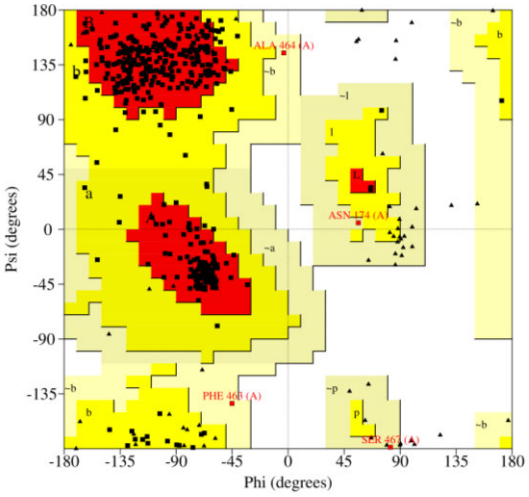
with a Grand average of hydropathicity (GRAVY) score of -0,060, suggesting strong interaction with water. This is crucial as it broadens ways of administration of the vaccine and can be introduced to chicken diet by addition to water.

4.4.7. 3D modelling, refinement, and validation of vaccine construct

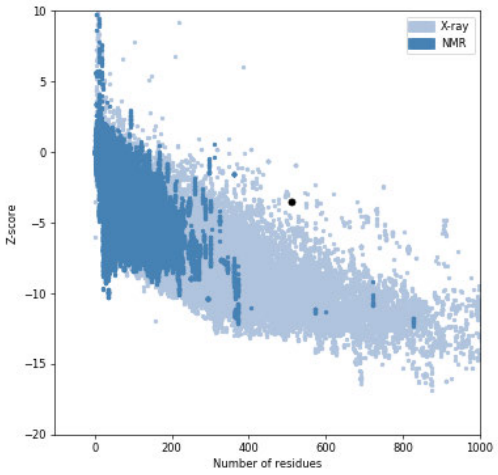
The designed multi-epitope vaccine's 3D structure was generated without any structural template using the trRosetta server. The server produced five different potential models which were subjected to GalaxyRefine server for protein structural refinement (Figure 4.2a). Amongst the models generated, Model 1 was chosen as potential vaccine structure and was further validated using the PROCHECKER server with Ramachandran plot percent of 83.6%, 12.4% and 1.0% of residues present in the favoured and allowed region (Figure 4.2b). It was noted though Ramachandran plot that structural refinement did improve the overall quality of the protein. The ProSA- web revealed a Z-score of -3.48, indicating the designed vaccine protein structure as a good quality since it lies within the score range of PBD X-ray experimental structures (Figure 4.2c).



(a)



(b)



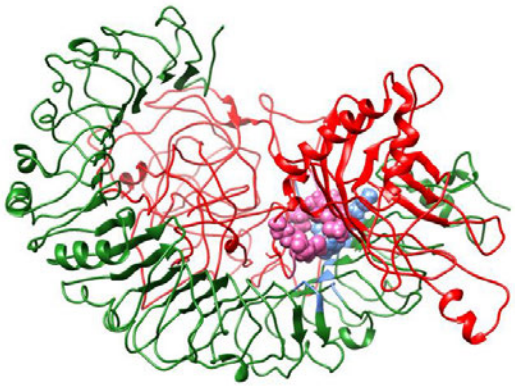
(c)

Figure 4.2: A validation analysis designed multiepitope vaccine. (a) Refined multiepitope vaccine construct (with adjuvant-red, linkers-orange, T-cell epitopes-cyan and yellow); b) Ramanchandran plot of refined protein structure with 83.6% of residues present the in favoured regions and c) ProSa web results showing Z score= -3.48.

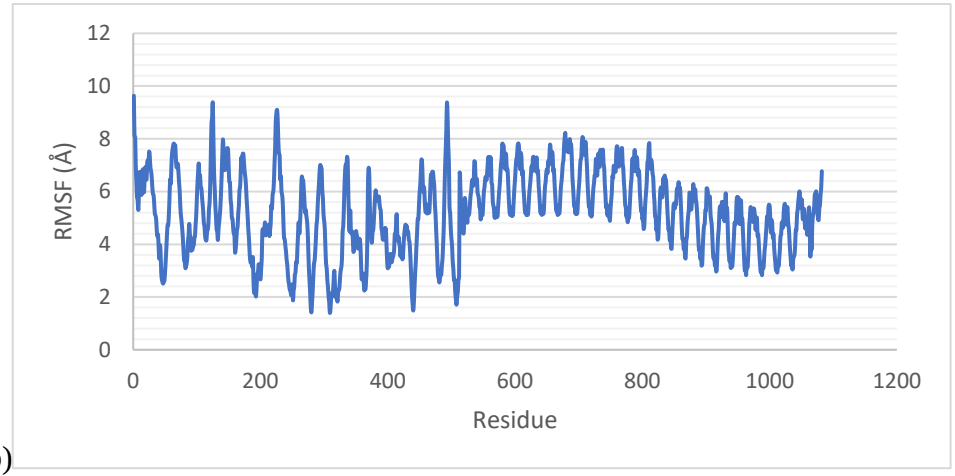
4.4.8. Molecular dynamics simulation of vaccine construct

The molecular dynamic simulation was performed to evaluate interaction affinity between vaccine construct and TLR4 and overall structural stability of docked complex (Figure 4.3 a). The RMSF plot (Figure 4.3 b) demonstrated interaction between amino acids side chains with TLR5. The plot revealed fluctuated regions, with high peaks observed at 100-300 residues and 400-600 residues. These observed fluctuated regions indicate high flexibility of the complex. The structural stability was assessed according to obtained RMSD and RMSF plots. The results observed from RMSD plot (Figure 4.3 c) revealed fluctuations in the docked complex began

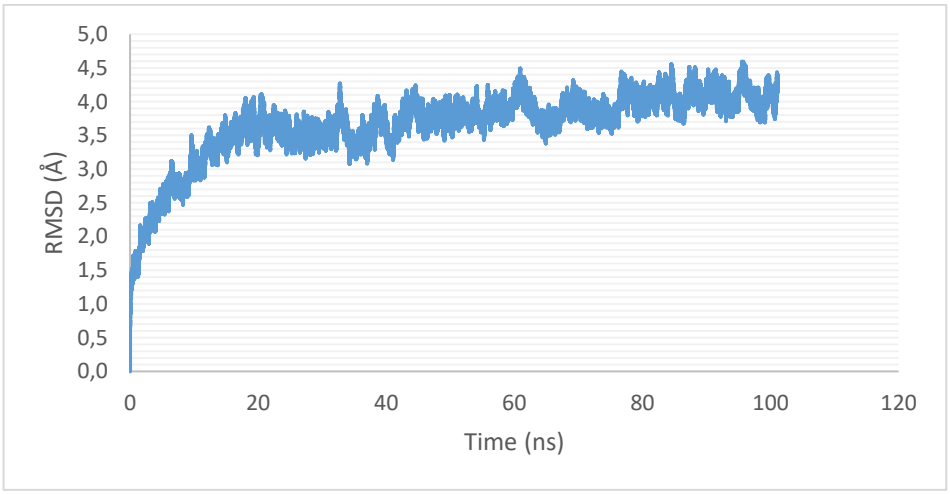
at 1.5Å and completed at 4Å. The vaccine reached a relatively stable position at 20 ns, where minor fluctuations were observed until completion of simulation.



(a)



b)



c)

Figure 4.3: Molecular dynamic simulation at 100 ns of vaccine construct and TLR4 (a) Docked complex of vaccine construct (red) with TLR4 (green). Vaccine construct residues interacting with TLR4 residues(blue) represented by hot pink, (b) RMSF plot indicating fluctuation and stability of amino acid residues in the side chains of docked complex. and (c) RMSD plot

representing structural stability of amino acid backbone between vaccine and TLR4 as a docked complex.

4.4.9. In silico codon optimization, cloning and expression of vaccine construct

Codon optimization of the peptide sequence of multiepitope vaccine construct generated an improved nucleotide sequence of vaccine with a CAI value of 1.0 and GC-content of 54.94 indicating optimal expression of the vaccine in the *E. coli* K12 strain. Finally, XhoI and BamHI restriction sites were introduced into the final vaccine and cloned into the expression vector petDEST42 (7336 bp construct) before *in silico* cloning and expression using SnapGene software (Fig. 4.4).

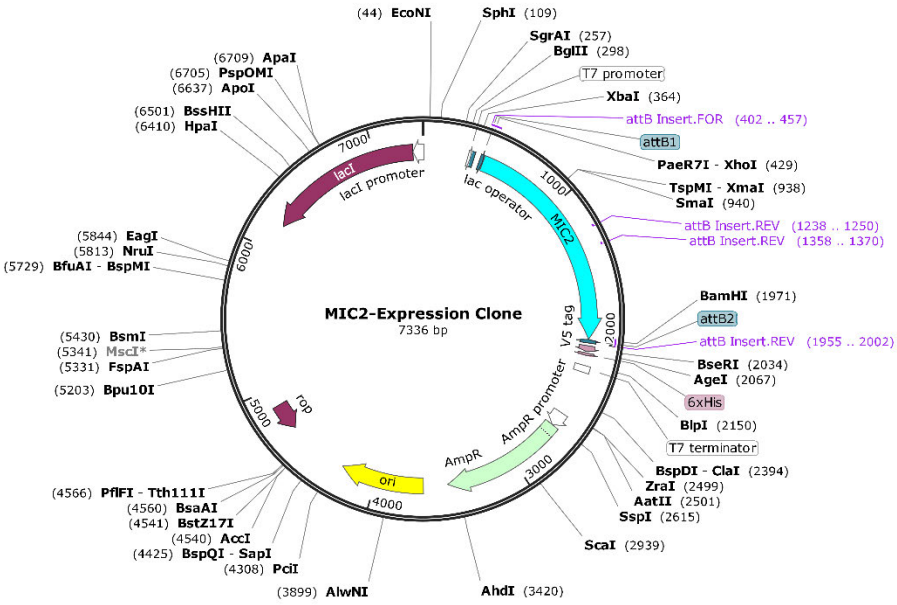
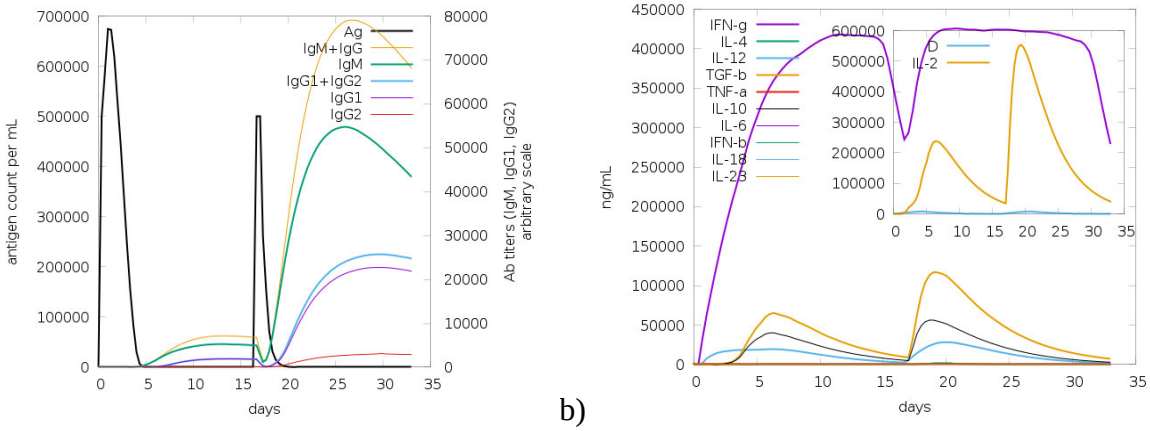


Figure 4.4: *In silico* cloning map for improved multiepitope sequence into petDEST42 vector, showing multiepitope vaccine labelled as MIC2 (cyan) connected with XhoI (429) at C terminal and BamHI (1971) at N terminal.

4.4.10. Vaccine candidate in silico simulations

The immune simulation of the designed vaccine was performed using C-ImmSim online server yielded results consistent with findings obtained in the prediction section. The administration of vaccine in the simulation exhibited a significant increase in cytokines such as IFN-gamma, TGF-b, IL-10, IL-23, and IL-12 indicating good immune response, correlating with the prediction of IFN-gamma and Interleukin inducing epitopes present in the vaccine (Figure 4.5b). The administration of vaccine revealed immune profiles associated with induction of

immune response, characterized by significant increase in tertiary immune response was exhibited by high levels of IgG1 + IgG2, IgG + IgM, IgG1 and IgG2 antibodies compared to primary response represented by IgM and IgG, upon recognition of antigen (Figure 4.5a). subsequent doses of vaccine into the simulation reduced concentration of antigen, while B-cell population showed constant increased levels, developing memory cells (Figure S4.1a). The decline in antigen levels confirmed the presence of antibodies that effectively maintained the possibility of an antigenic surge.



a) b)

Figure 4.5: immune simulation of multiepitope vaccine construct. (a) immunoglobulin production in response to antigen injections; (b) cytokine level induced by vaccine treatment given over 4 weeks apart

4.5. DISCUSSION

Avian coccidiosis continues to be the world's most widespread and severe gastrointestinal infectious disease in poultry, exerting high economic losses to the poultry industry (Blake et al., 2017; Dalloul and Lillehoj, 2006). Currently, most commercial vaccines available to treat coccidial parasites are derived from live virulent or attenuated organisms. With reported concerns over the huge costs lost on production and shelfing of these vaccines (Atapour et al., 2019; Saha and Raghava, 2006), rigorous research on alternative control strategies that are cost efficient and have long shelf life is required. The use of peptide-based subunit vaccines as an alternative to drugs is currently an attractive option to combat infection with *Eimeria*. Hence, this study's main objective was to develop an effective vaccine against *Eimeria* by prediction of multi-epitope vaccine candidates using immunoinformatic and reverse vaccinology strategies, that could induce long lasting immunity against coccidiosis.

In the present study, the conserved sequences of MIC2 antigen were selected and used to generate antigenic and immunogenic T-cell epitopes. Transmembrane analysis of the conserved sequences revealed the strong transmembrane potential of MIC2 epitopes, crucial for pathogenesis (Dar et al., 2019). The selection of epitopes was based on antigenic and immunogenic properties using bioinformatics tools, which enabled prediction of 7 $CD8^+$ T-cell epitopes and 12 $CD4^+$ T-cell epitopes capable of inducing IFN- γ and IL-4 cytokines. T-cell induced cytokines exhibit immunoregulatory effect on host and hinders development of the parasite within host (reviewed by Kim et al., 2019), affirming presence of such epitopes in the vaccine construct ensured the stimulation of humoral and innate responses and a vaccine's ability to confer complete protection against coccidiosis. The inclusion of the adjuvant Monophysphoryl lipid A(MPLA) enhanced immunogenicity (score of 9.419) and efficiency of the vaccine in eliciting an immune response. This adjuvant was used previously by Song et al. (2017) where inclusion of MPLA adjuvant with toxofilin DNA vaccine against *T. gondii* stimulated highly enhanced humoral and Th1-biased immune responses by increased production of IgG.

Toll- like receptors such as TLR4 are associated with pathogen detection and reduction through stimulation of host responses (Zhang et al., 2012; Zhou et al., 2013). Presence of TLRs such as TLR4 and TLR5 in host were reported to stimulate the expression of cytokines crucial for innate immune response to *E. tenella*. such as interferons (IFNs), tumour necrosis factor (TNF) and IL-6, which is an imperative factor in vaccine development. Molecular docking of the designed multiepitope with TLR4 showed significantly strong interactions, suggesting capability of the designed vaccine to induce efficient immune response against *Eimeria*. This was confirmed by molecular dynamic simulation of the docked complex, which exhibited high flexibility and stability (Figure 4.3b), indicating stable binding of vaccine and TLR. Codon optimization results showed the vaccine to have CAI value and GC content of 1.0 and 54.95 respectively, indicating improved expression of vaccine in a vector. The observed findings are similar to a separate study by Nourmohammadi et al. (2020) where multi-epitope vaccine against cystic echinococcosis was designed using EgA31 and EgG1Y162 antigens.

Immune simulation exhibited outcomes consistent with a typical immune response due to repeated exposure to an antigen. The generation and increase of secondary and tertiary responses were marked by high levels of IgG1 + IgG2 and IgG + IgM antibodies (Figure 4.5a). Similar findings were noted by Shey et al. (2019), where the authors observed secondary and tertiary responses represented by high levels of antibodies compared to antigen concentration,

indicating host's protection against disease. The gradual increase of memory B-cells and antibodies after subsequent exposure to three vaccine doses confirmed positive effects exhibited due to prolonged exposure of host to vaccine. The production of cytokines such as interleukin (IL)-12, 23 and interferon (IFN)-γ is critical for host innate immunity against *Eimeria* infections. The presence and maintained high level of these cytokines after initial administration of vaccine indicates abundance of TH cells, supporting a humoral response.

The vaccine was further evaluated for physiochemical and allergenicity characteristics to assess the quality of the vaccine construct. The designed construct was projected as stable, immunogenic, non-allergenic and hydrophilic, desired characteristics for vaccine suited for production. Molecular docking of the constructed multiepitope with TLR4 was done to assess its interaction with receptors for an enhanced immune response (Keestra and Putten, 2008). This vaccine candidate predicted and designed by in silico tools should be further validated through experimental studies.

4.6. CONCLUSION

This study used reverse vaccinology and bioinformatic approach to predict novel MIC2 antigenic T-cell epitopes (7 $CD8^+$ and 19 $CD4^+$) and designed a peptide-based vaccine against *Eimeria*. The vaccine constructed consisted of 512 amino acids and was enhanced by the addition of MPLA adjuvant for improved immunogenicity. The projected characteristics of the designed vaccine demonstrate the potential of the predicted epitopes in controlling coccidiosis by binding to the MIC2 antigen, hindering parasite invasion. The use of this vaccine candidate promises the induction of immune cellular and humoral response, and potentially conferring effective protection on the host. The inclusion of an MPLA adjuvant to the vaccine construct significantly enhanced immunogenicity of the vaccine and its ability to potentially stimulate immune protection against *Eimeria* challenge. It is highly recommended that further experimental validation studies should be done on the current study results to confirm the efficacy of the vaccine.

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CHAPTER FIVE

5.0. GENERAL DISCUSSION

The dire economic loss inflicted by chicken coccidiosis on the poultry industry and rising concern over negative impact resulting from administration of chemoprophylaxis therapy on chickens has encouraged exploring various alternatives to serve as preventive measures against coccidiosis. The economic loss experienced in poultry due to a significant decline in productivity and welfare of chickens resulting from *Eimeria* infections is estimated to excess US$ 13.6 billion, worldwide (Blake et al., 2020). Due to time, labour, and cost constraints associated with the design of vaccines, computational vaccinology is being considered for the prediction and design of vaccines against various infectious diseases (Magdeldin et al., 2012). Computational design of T-cell epitopes is highly imperative in vaccine development as it promises to facilitate mass production of vaccines with long shelf life, at low cost.

In the present study, vaccine design for both IMP1 and MIC2 antigens was achieved by identifying antigenic and immunogenic epitopes using various immunoinformatic tools. The predicted epitopes were assessed for overlapping ability ($CD8^+$), IFN-gamma and IL-4 inducing properties ($CD4^+$) and effectiveness to bind to selected chicken B-F MHC alleles. The overlapping of T-cells assured stimulation/ activation of cell-mediated response (Zhang et al., 2020) The effectiveness of these predicted epitopes heavily relied on the accuracy of bioinformatic tools used for prediction. T-cell epitope prediction was achieved using IEDB prediction server, with human leukocyte antigen (HLA) alleles chosen as a substitute to chicken alleles. Similar tools were used by Pandey et al. (2018) when predicting CTL and HTL epitopes for multi-epitope vaccine subunit vaccine for malaria. Due to limitation in data regarding chicken MHC alleles and as target species, some studies focusing on chicken have used NetMHCcons and NetMHCpans server for epitope prediction, selecting human HLA alleles determined as suitable chicken substitutes (Higgs et al., 2006) Regardless of different protocols used for prediction, both servers proved accurate. Selection of specific antigenic and immunogenic T-cell epitopes in a parasite antigen, may enable host to exhibit focused immune response projected to important epitopes, limiting non-protective response or autoimmunity which may result if whole parasitic antigens are incorporated into a vaccine (Awadelkareem et al., 2020).

The vaccines designed for both antigens were found to be antigenic, thermostable, and non-allergic. Some differing outcomes noted for both vaccines were in GRAVY and pI, where

IMP1 vaccine construct was found to be slightly acidic (pI = 6.33) and hydrophobic with GRAVY score of 0.209 (Chapter 3). The MIC2 vaccine was detected to be basic (pI =10.26) and hydrophilic (GRAVY = -0.060), suggesting strong interaction with water molecules. The difference in hydrophobicity (GRAVY) suggests possible structural or location differences between the antigens, where IMP1 construct consisted of mostly membranous epitopes, while MIC2 is made of globular epitopes (Pandey et al., 2018). The hydrophobic nature of IMP1 multiepitope vaccine also suggests the epitopes making up the construct are located on the interior of protein, driving stableness of the protein (Khatoon et al., 2018). These results correlate with the previous characterization of both antigens (Jenkins et al., 2015; Yan et al., 2018). Proteins secreted from sporozoite MICs are suggested to play a vital role in parasite attachment and host cell invasion by facilitating interaction between parasites and host cell epitopes. (Higgs et al., 2006; Zhang et al., 2020) confirming the importance of including epitopes or antigens associated with these organelles in the design of vaccines for protection against infectious disease.

The use of TLRs serves crucial in the activation of adaptive immune response and innate response through regulating the expression of cytokines and chemokines(Higgs et al., 2006; Keestra and van Putten, 2008). TLRs are highly conserved molecules that detect conserved pathogen material in opportunistic microorganisms. In the present study, IMP1 and MIC2 epitope-based vaccine constructs were docked with TLR5 (flagellin) and TLR4, respectively. The molecular docking with receptors revealed strong interaction presented by high binding affinities. Several studies have used both receptors against varying diseases to successfully activate immune responses (Gupta et al., 2014; Yin et al., 2013). Interaction of TLR and appropriate ligands initiate the production of various inflammatory cytokines and interferons (IFN) that regulate the acquired immune response (Pandey et al., 2018); hence, strong interaction observed from binding of TLR with predicted epitopes will enhance effectiveness and stability of designed vaccines.

This was further confirmed when the designed vaccine was subjected to immune simulation, where administration of vaccine stimulated production of antibodies correlating to tertiary responses while controlling antigen concentrations. Subsequent administration of vaccine aided in production or upregulation of IFN- γ, IL-2 and IL-12 associated with innate immunity against *Eimeria* challenge. Codon adaptation results showed improved expression of designed multiepitope constructs with CAI values of 1 and GC content of 55.19 (IMP1) and 54.95 (MIC2) in *E. coli* strain K12 system. This suggests that designed vaccines have the potential to be adapted for transfection through cloning to any suited expression vectors ideal for delivering vaccine to host. The immoinformatics and various *in silico* validation approaches applied in the current study for design of multiepitope vaccine provide a highly imperative and effective option for vaccine development. They may be the long-needed alternative for prediction and design of multiepitope peptide vaccines at a large scale before experimental validation (Khan et al., 2020).

5.1 CONCLUSION AND RECOMMENDATIONS

To curb the negative effect of coccidiosis on chicken welfare and the poultry industry due to losses in costs from chicken mortality and treatment measures to control the disease, exploration of novel and effective alternatives is imperative. Recombinant DNA vaccines have exhibited promising outcomes against coccidiosis by providing protection of the host. In the present study, the prediction and design of multiepitope-based vaccine candidates from 2 *Eimeria* antigens was achieved through a series of immunoinformatic tools. A total of 7 and 21 T-cell epitopes were predicted for IMP1 antigen, and 7 and 19 T-cells ($CD8^+$ and $CD4^+$) were successfully predicted for MIC2 antigen. This led to the design of two multi-epitope vaccine constructs that are high conserved, antigenic, immunogenic, stable, and non-allergic. The designed vaccines exhibited highly desired physiochemical and immunological properties crucial for stimulating immune responses against *Eimeria* infections. The designed vaccines promise effective induction of cellular and humoral immune response, conferring effective protection on the host. From findings noted in an immune simulation study of the vaccine, T-cell epitopes may be best-suited vaccine candidates, showing potential in inducing long term response within the host. It can be concluded that exploration of parasite antigens using immunoinformatic approaches to design multi-epitope vaccine yielded promising results. However, with an average accuracy of some tools used for epitope predictions, it is highly recommended that further studies and experimental validation should be done on the results obtained and reported in the current study to verify and validate safety and efficacy of the vaccine candidates.

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APPENDIX

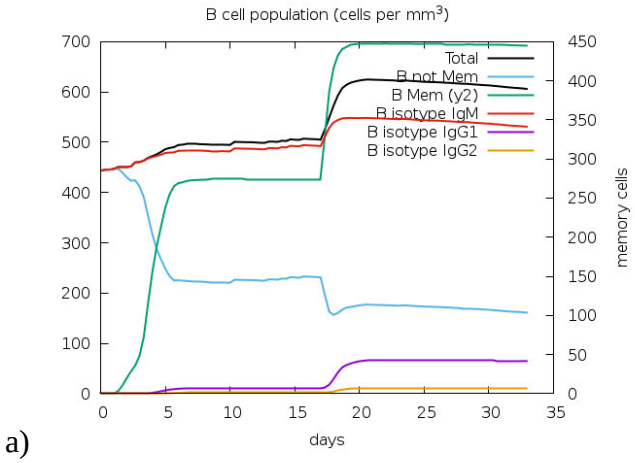
8.1. Supplementary material for IMP1 (chapter 3)

Table S3.1: Details of five best refined vaccine models generated by GalaxyRefine web.

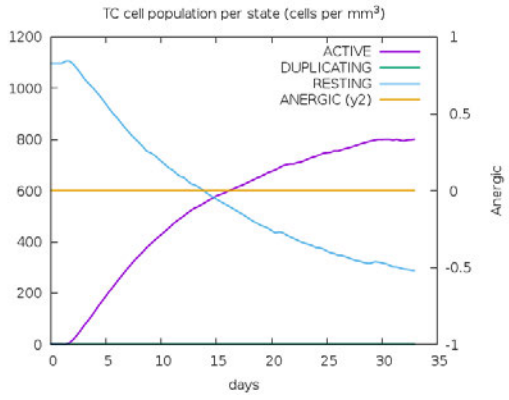
Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored
Initial	1.0000	0.000	5.106	342.9	82.9	84.9
MODEL 1	0.8598	0.636	3.351	83.1	3.3	89.3
MODEL 2	0.8578	0.640	3.260	83.2	2.6	89.5
MODEL 3	0.8615	0.635	3.432	87.4	3.8	88.8
MODEL 4	0.8639	0.634	3.370	82.9	3.8	90.3
MODEL 5	0.8684	0.626	3.399	84.1	3.8	89.5

Table 3.2: C-ImmSimm parameters for designed multiepitope vaccine immune response simulation

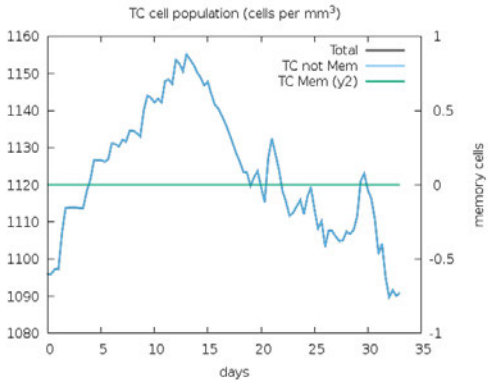
Parameters	
Rand seed	12345
Simulation volume	10
No. of steps	100
No. of injections	3
Time of injection	1,50,100
Antigen to inject	1000



a)



b)



c)

Figure S3.1: immune simulation showing immune response induced by designed vaccine through a) The evolution of B-cell population after three injections; b) Evolution of T-cytotoxic cell populations per state and cells per mm^3 after subsequent injections.

8.2. Supplementary material for MIC2 antigen (Chapter 4)

Table S4.1: Structural information of the Best 5 refined models of chosen model 1 of vaccine

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored
Initial	1.0000	0.000	2.396	16.7	1.6	90.8
MODEL 1	0.9741	0.333	2.127	14.6	0.8	92.7
MODEL 2	0.9756	0.334	2.175	15.1	1.0	92.2
MODEL 3	0.9702	0.353	2.151	14.4	1.0	92.4
MODEL 4	0.9746	0.333	2.087	12.9	0.3	92.5
MODEL 5	0.9644	0.376	2.186	16.0	0.5	92.2

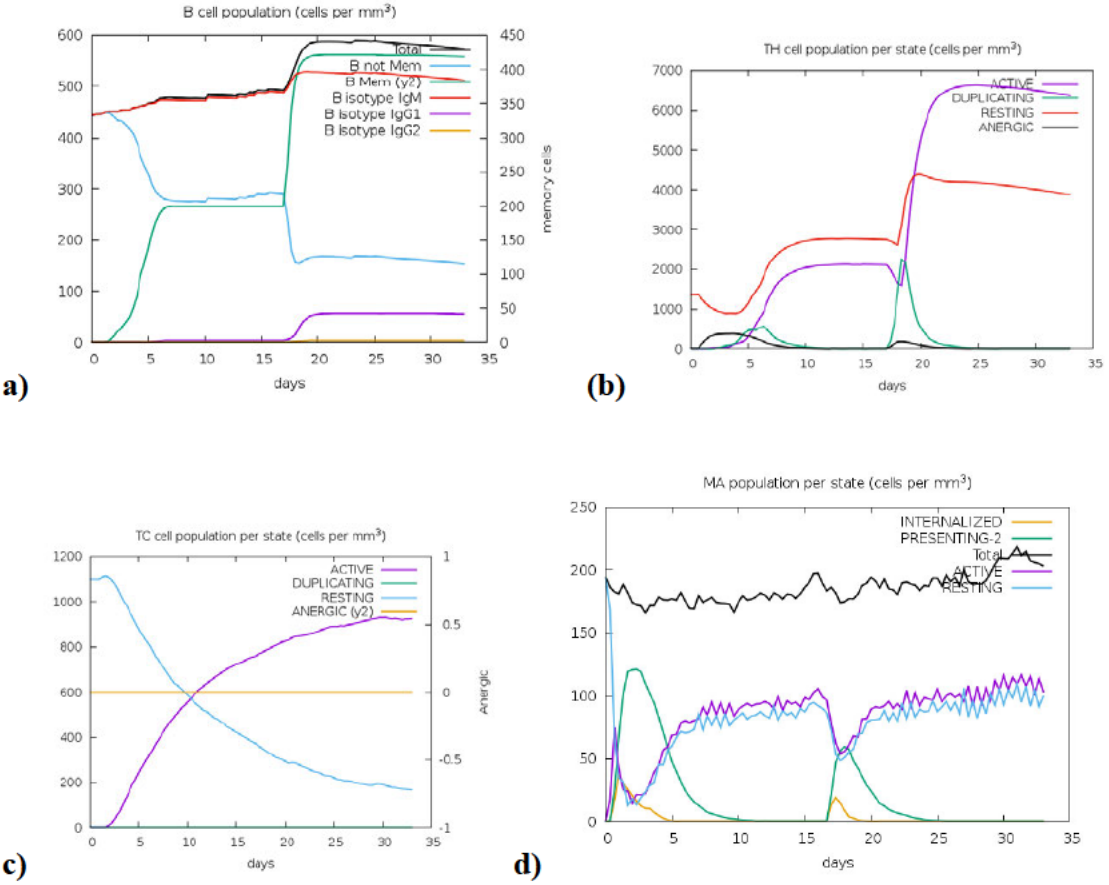


Fig S4.2: immune simulation response of multiepitope vaccine. (a) Growth of B-lymphocytes and memory cells; (b) evolution of $CD4^+$ T-helper lymphocytes; (c) $CD8^+$ T-cytotoxic lymphocytes count per entity-state; and (d) growth of Macrophages, presenting on MHC class-II, active and resting macrophages

Weblink References

[1] https://www.ncbi.nlm.nih.gov/protein
[2] http://www.genome.jp/tools-bin/clustalw
[3] http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html
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