UNIVERSITI PUTRA MALAYSIA

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SERDANG, SELANGOR
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SYAHIR AIMAN BIN SHAHRIL AGUS

A project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia
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FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA

December 2023

CERTIFICATION

It is hereby certified that I have read this project paper entitled “Effect of N, N – Dimethylglycine (DMG) Supplementation on Haematological Parameters and Frequency of CD4+ and CD8+ T Cells in Cats Post-vaccination” by Syahir Aiman Bin Shahril Agus (200749) and in my opinion, it is satisfactory in terms of scope, quality, and presentation as a fulfilment of the requirement for the course of VPD4999 Final Year Project.

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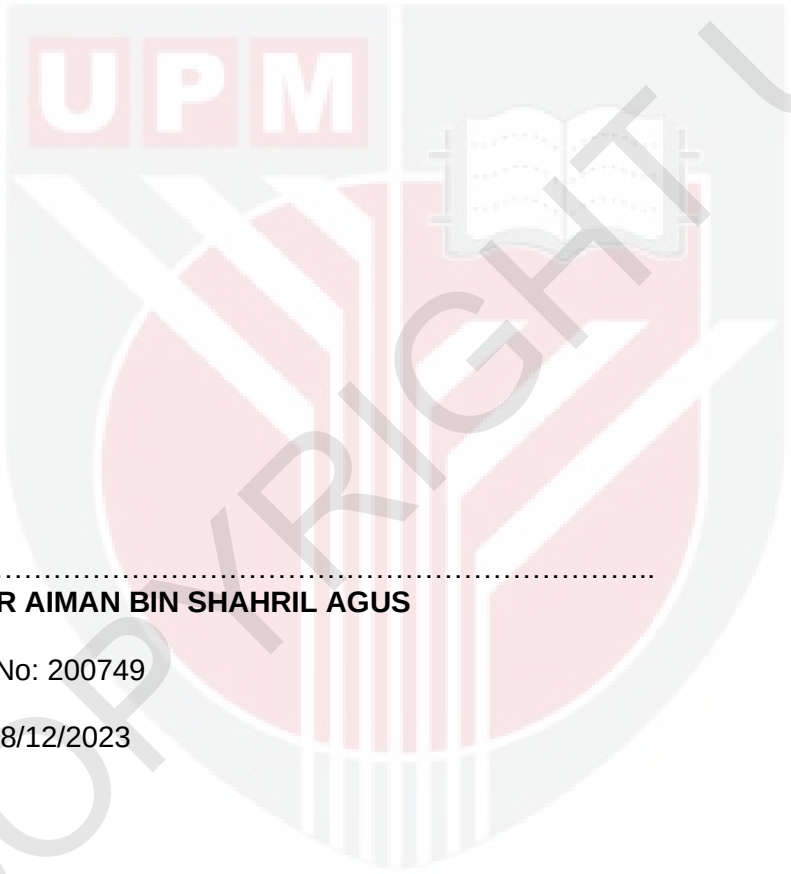
DECLARATION

I hereby declare that this thesis is based on my original work except for the quotations, illustrations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at Universiti Putra Malaysia or other institutions.



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LIST OF ABBREVIATIONS

AAFP	American Association of Feline Practitioners
BAS	Basophil
CAM	Complementary and alternative medicine
CMI	Cell mediated immunity
DNA	Deoxyribonucleic acid
DMG	Dimethylglycine
EOS	Eosinophil
FBS	Fetal bovine serum
FCV	Feline Calicivirus
FHV-1	Feline Herpesvirus 1
FITC	Fluroscein isothiocynate
FIV	Feline Immunodeficiency Virus
FMO	Fluorochrome minus one
FPV	Feline Panleukopenia Virus
FSC	Forward scatter
HCT	Haematocrit
HGB	Haemoglobin
IQR	Interquartile range
LYM	Lymphocyte
MON	Monocyte
NEU	Neutrophil
PBS	Phosphate buffered saline
PE	Phycoerythrin
PLT	Platelet

PRR	Pattern-recognition receptors
RBC	Red blood cells
SSC	Side scatter
WBC	White blood cells
WSAVA	World Small Animal Veterinary Association

ABSTRAK

Abstrak daripada kertas project yang dikemukaan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 Projek Ilmiah Tahun Akhir

EFEK SUPLEMENTASI N, N-DIMETILGLISIN (DMG) TERHADAP PARAMETER HEMATOLOGI DAN KEKERAPAN SEL T CD4+ DAN CD8+ KUCING PASCA-VAKSINASI

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ABSTRAK

N, N-Dimetilglicin (DMG) merupakan asid amino tertier yang biasanya digunakan sebagai nutraseutikal dalam perubatan veterinar. Ia dikatakan mempunyai sifat imunomodulasi seperti menggalakkan penghasilan antibodi dan limfosit. Dengan ketara, kajian terkini mengenai topik ini dalam kucing masih kurang, dengan kajian terakhir dilakukan pada tahun 1992. Kajian novel ini mengkaji kesan suplementasi DMG terhadap parameter hematologi kucing dan peratusan sel T CD4+ dan CD8+ selepas pemberian satu dos vaksin Purevax®Feline 4 yang mengandungi virus hidup terubah suai rhinotracheitis-calici-panleukopenia dan *Chlamydia psittaci*. Dua belas kucing yang telah dimandulkan dari sebuah tempat perlindungan haiwan, yang terdiri daripada kedua-dua jantina, dibahagikan kepada kumpulan kawalan (n=6) dan kumpulan rawatan (n=6), dan diberi satu dos vaksin pada hari ke-0. Kumpulan rawatan menerima suplemen DMG secara oral (125 mg/ml) pada dos 0.5 ml dua kali sehari selama 14 hari dan juga diberi satu dos vaksin. Sampel darah diambil pada hari ke-0 (sebelum rawatan) dan hari ke-14 (selepas rawatan), dan analisis

hematologi dijalankan untuk menilai parameter seperti jumlah sel darah merah, jumlah trombosit, dan peratusan jenis sel darah putih. Sel darah mononuklear periferi diasingkan dan dianalisa menggunakan *sitometri aliran* untuk menentukan peratusan sel T CD4+ dan CD8+. Analisis statistik dijalankan menggunakan ujian T bukan parametrik untuk mengenal pasti sebarang perbezaan yang signifikan. Analisis hematologi mendapati bahawa jumlah sel darah merah, hematokrit, hemoglobin, jumlah trombosit, jumlah sel darah putih serta peratusan monosit, eosinofil, dan basofil tidak menunjukkan perbezaan yang signifikan ($P>0.05$) di antara kumpulan kawalan dan rawatan selepas 14 hari eksperimen. Menariknya, pada hari ke-14 selepas rawatan, peratusan neutrofil kumpulan rawatan adalah lebih rendah secara signifikan ($P=0.0238$) berbanding dengan kumpulan kawalan, manakala peratusan limfosit kumpulan rawatan adalah lebih tinggi secara signifikan ($P=0.013$) berbanding dengan kumpulan kawalan. Bagi peratusan subkelompok sel T, terdapat peningkatan yang signifikan ($P=0.0022$) dalam peratusan CD4+ dalam kumpulan kawalan dari pra-rawatan hingga selepas rawatan, manakala ini tidak dilihat dalam kumpulan rawatan. Kedua-dua kumpulan menunjukkan penurunan dalam peratusan CD8+ dari pra-rawatan hingga selepas rawatan, namun ia tidak signifikan secara statistik. Akhirnya, nisbah CD4:CD8 kedua-dua kumpulan tidak menunjukkan perbezaan yang signifikan apabila membandingkan nilai pra-rawatan dengan nilai selepas rawatan. Kesimpulannya, suplementasi DMG mungkin mempunyai kesan yang signifikan dalam pengurangan peratusan neutrofil dan peningkatkan peratusan limfosit. Sebaliknya, suplementasi DMG kelihatan tidak memberi impak yang signifikan dalam mempengaruhi peratusan subkelompok sel T terutamanya CD4+ dan CD8+. Keputusan ini menekankan keperluan untuk penyelidikan lebih lanjut untuk memahami dengan lebih baik kesan DMG terhadap kesihatan dan imuniti kucing.

Kata Kunci: Dimetilglicin, imunomodulasi, parameter hematologi, CD4+, CD8+, kucing

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 Final Year Project

EFFECT OF N, N-DIMETHYLGLYCINE (DMG) SUPPLEMENTATION ON HAEMATOLOGICAL PARAMETERS AND FREQUENCY OF CD4+ AND CD8+ T CELLS IN CATS POST-VACCINATION

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ABSTRACT

N, N-Dimethylglycine (DMG) is a tertiary amino acid that is commonly used as a nutraceutical in veterinary medicine. It is claimed to have immunomodulating properties such as supporting the production of antibodies and lymphocytes. Notably, recent studies on this topic in cats are lacking, with the last study dating back to 1992. This novel study investigated the effect of DMG supplementation on feline haematological parameters and the percentage of CD4+ and CD8+ T cells after vaccination with single dose of *Purevax®Feline 4* core vaccine containing modified live rhinotracheitis-calici-panleukopenia virus and *Chlamydia psittaci*. Twelve neutered cats from an animal shelter of both genders were divided into control (n=6) and treatment groups (n=6), and were given one dose of vaccination at day 0, with the latter receiving oral DMG supplementation (125mg/mL) at a dosage of 0.5 mL twice daily for 14 days alongside vaccination. Blood samples were collected on day 0 (pre-treatment) and day 14 (post-treatment), and haematological analysis was performed to evaluate parameters such as complete blood count and differential leukocyte count. Peripheral blood mononuclear cells were isolated and subjected to

flow cytometry to determine CD4+ and CD8+ T cell percentages. Statistical analyses were conducted using non-parametric T test to identify any significant differences. Haematological analysis revealed that red blood cell, haematocrit, haemoglobin, platelet, white blood cell counts as well as monocyte, eosinophil, basophil percentages showed no significant difference (P>0.05) between control and treatment group after 14 days of experiment. Interestingly, on post treatment (day 14), neutrophil percentage of the treatment group was significantly lower (P=0.0238) compared to the control group while lymphocyte percentage of the treatment group was significantly higher (P=0.013) compared to the control group. As for T cell subset percentages, there was a significant increase (P=0.0022) in CD4+ percentages in the control group from pre-treatment to post-treatment while this was not seen in the treatment group. Both groups exhibited a decrease in CD8+ percentage from pre-treatment to post treatment, however, it was not statistically significant. Lastly, CD4:CD8 ratios of both groups showed no significant difference when comparing pre-treatment values to post-treatment values. In conclusion, DMG supplementation may have a significant effect in decreasing neutrophil percentage and increasing lymphocyte percentage. In contrast, DMG supplementation appears to have no significant impact in influencing T cell subsets percentages particularly CD4+ and CD8+. These results emphasize the need for more research to better understand DMG's effects on feline health and immunity.

Keywords: Dimethlyglycine, immunomodulation, haematological parameters, CD4+, CD8+, feline

CHAPTER 1

INTRODUCTION

1.1 Background

Nutraceuticals can be defined as food products or components of food that are postulated to be beneficial for human and animal health (Hayek et al, 2004). An example of a nutraceutical that is commonly used in veterinary medicine is N, N-Dimethylglycine (DMG). DMG is a naturally occurring tertiary amino acid and a by-product of the metabolism of choline (Lawson et al., 2007). The immunomodulating properties of DMG has been examined in multiple studies in the past and the results are variable. DMG is claimed by its proponents to be able to support the production of both lymphocytes and antibodies. According to studies done on humans by Graber et al. (1981), human subjects that were given DMG orally, exhibited four times increase of antibodies after vaccination with pneumococcal vaccine compared to human subjects in the control group. Similarly, in a study done on rabbits, animals that are supplemented with DMG showed four times increase of antibody titre towards influenza antigen post vaccination with a killed influenza virus vaccine (Reap & Lawson, 1990). Interestingly, in contrast, DMG failed to exhibit its immunomodulating capabilities in a cat study. In a study conducted by Weiss (1992), cats treated with DMG showed a lower titre of virus neutralizing antibodies against feline herpesvirus-1 when compared to the control group. In the same study, there was no significant difference in lymphocyte blastogenic response to phytolectins between leukocytes incubated with DMG in-vitro and untreated leukocytes. Given that the last published research investigating DMG's immunomodulating properties in the feline species dates back to 1992, there is an urgent need to revisit and study this topic in order to update our understanding and potentially harness its benefits for feline health.

1.2 Justification

The immunomodulating capabilities of N, N-Dimethylglycine (DMG) has been studied in species such as horses (Beech, 1987), rabbits (Reap & Lawson, 1990), humans (Graber et al., 1981), and even cats (Weiss, 1992). However, results from these studies are mixed and no consensus has been reached regarding DMG's immunomodulating properties. Currently as of 2023, there are no recent studies on the effect of DMG on complete blood count parameters and percentage of CD4+ and CD8+ T cells from Peripheral Blood Mononuclear Cells (PBMC) of cats. Since this is a significant gap in research, therefore, our project aims to investigate the effects of DMG on these parameters in cats. We hoped that the data obtained from this study can help veterinarians to make informed decisions regarding the usage of DMG as a health supplement particularly as an immune booster.

1.3 Objectives

1. To examine the effects of N, N-Dimethylglycine (DMG) supplementation on haematological parameters in cats after vaccination.
2. To determine the percentage of CD4+ and CD8+ T Cells from Peripheral Blood Mononuclear Cells (PBMC) post supplementation with DMG after core vaccination.

1.4 Hypothesis

For this study, the null hypothesis (H_0) is there is no significant difference in the haematological parameters and/or percentage of CD4+, CD8+ T cells between cats supplemented with DMG and control cats. In contrast, the alternative hypothesis is there is a significant difference in the haematological parameters and/or percentage of CD4+, CD8+ T cells between cats supplemented with DMG and control cats.

CHAPTER 2
LITERATURE REVIEW

2.1 Overview of Feline Immune System

All animals including cats are exposed to a plethora of pathogens including bacteria, virus and parasites on a daily basis. As a result, animals have evolved to develop a specialized system called an immune system to protect themselves against these pathogens. The immune system consists of a network of tissues, cells and biomolecules that interacts with one another via hormone-like substances called cytokines with the main goal of protecting the host from pathogens as well as malignant cells and toxins (Hayek et al, 2004). In general, the feline immune system functions the same and it is very similar structurally to other placental mammals (Tizzard, 1998). Essentially, the immune system can be categorized into two functional subsystems called the innate immune system and acquired (adaptive) immune system (Callahan & Yates, 2014). The innate immune system which consists of cells such as sentinel cells (macrophages and dendritic cells), phagocytes (macrophages and neutrophils) and natural killer cells provide the host with a non-specific, quick and early, albeit short-lived response to infectious threats (Callahan & Yates, 2014). The innate immune system is triggered when the sentinel cells' pathogens pattern-recognition receptors (PRR) bind to specific ligands on the surface of invading microorganisms which causes these sentinel cells to release cytokines in order to recruit phagocytes to neutralize the invading pathogen (Callahan & Yates, 2014). Unlike the acquired immune system, innate immune system lacks memory of the initial encounter with the invading pathogen which translates to no increase in efficiency in neutralizing the same pathogen despite multiple encounters in the future (Callahan & Yates, 2014). As for the acquired immune system, the main cells involved in this system includes T cells and B cells. T cells can be further categorized to CD4+

T cells also known as T helper cells that play a role in activation of B cells to produce neutralizing antibodies and CD8+ T cells also known as T cytotoxic cells that directly causes the destruction of virus-infected cells via the induction of apoptosis. (Hayek et al, 2004). In the feline species, roughly 40-45% of lymphocytes in the peripheral blood are B cells and 32-41% of lymphocytes in the peripheral blood are T cells (Tizzard, 1998). A key characteristic of the acquired immune system is its ability to remember specific antigen from the initial encounter via the production of highly specialized memory T and B cells that remain in circulation long after the initial infection has subsided. As a result, future encounters with the same antigen will result in a faster, and stronger immune response by T cells and B cells (Callahan & Yates, 2014).

2.2 Immune Response Induced by Vaccination

Based on multiple guidelines and recommendations by various companion animal veterinary association such as American Association of Feline Practitioners (AAFP) and World Small Animal Veterinary Association (WSAVA), it is highly recommended that all cats should be vaccinated against the three core pathogens; feline panleukopenia virus (FPV), feline calicivirus (FCV) and feline herpesvirus-1 (FHV-1) (Day *et al.,* 2016; Stone *et al.,* 2020). On the other hand, vaccination against feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), and *Chlamydophila felis* are considered non-core vaccines. However, the FeLV vaccine can be considered a core vaccine for adult cats that are at-risk such as those with unsupervised outdoor access or those that live in a multiple cat household (Stone *et al.,* 2020). Currently, in Malaysia, cat vaccines are most commonly available in the form of polyvalent modified-live vaccines consisting of attenuated strains of FPV, FCV, FHV-1 and *Chlamydia psitacci.* Modified-live vaccines contain pathogens that are weakened artificially through the process of repeated passages of cell culture. Due to

this, modified live vaccines induces a more robust (humoral and cell mediated immunity) and faster onset of immune response compared to inactivated (killed) vaccines (Stone *et al.,* 2020). It is important to note that the immunity conferred by the vaccine differs from pathogen to pathogen. Vaccination against FPV will provide the cat with sterilizing immunity; a type of immunity that prevents both infection as well as development of clinical disease, and this type of immunity is best determined via measuring vaccine-induced antibody titres (Dodds, 2021). In contrast, for pathogens such as FCV and FHV-1, vaccination against these pathogens would only provide the animal with a non-sterilizing immunity which only prevents severe clinical disease in infected cats and not infection (Dodds, 2021). The immune response of cats against FCV vaccines and FHV-1 vaccine also differs from the immune response against FPV vaccine. For both FCV and FHV-1, there is no correlation between protection (immunity) and titres of antibodies, which signifies that cell mediated immunity (CMI) plays a more important role for protection against these pathogens. Indeed, this is true for FCV as studies have shown that despite the absence of virus-neutralizing antibodies, cats are being protected against FCV infection, indicative of protection that is reliant on CMI (Poulet *et al.,* 2008; Lesbros *et al.,* 2013). The same is true for FHV infection, where CMI plays a more vital role for protection compared to humoral immunity (Thiry *et al.,* 2009). Similarly, in a study conducted by Flynn *et al.,* (2000), cats that are vaccinated with FeLV DNA vaccine are protected against FeLV challenge despite having no FeLV-specific humoral immunity. Also in the same study, the elimination of the virus from infected cats occurred before the production of virus-specific antibodies, suggesting that the recovery of cats with temporary viremia was not primarily dependent on the action of humoral immunity. In summary, the feline immune response to vaccination varies according to the specific pathogen

targeted by the vaccine. Additionally, it is noteworthy that in certain diseases, like FCV and FHV-1 infections, CMI plays a more prominent role in providing protection.

2.3 Flow Cytometry and Immunophenotyping

Flow cytometry is a quantitative and qualitative method to rapidly analyze the characteristics of singular cells via the use of a single or multiple lasers (Brown & Wittwer, 2000). Typically, in a flow cytometer machine, samples are channeled to the interrogation point via highly pressurized buffered saline solution (sheath fluid) where lasers are then beamed directly into each individual cell or particles (McKinnon, 2019). The scattering of light that is produced after the laser has passed through the individual cells are detected by sensors and analyzed by a computer (McKinnon, 2019). Physical properties of each individual cells such as cell size and cell complexity (granularity) can be determined based on the forward scatter of light (FSC) and side scatter of light (SSC) respectively (Brown & Wittwer, 2000). A major application of flow cytometry is the ability to analyze the immune system particularly at the cellular level (Finak *et al.*, 2016) which applies to veterinary medicine as well. In a study conducted by Azizi *et al.* (2021), flow cytometry was employed for the evaluation of T cell immune response particularly CD4+ and CD8+ T cells in chicks after vaccination with a Fowl Pox vaccine. This is achievable via a technique called immunophenotyping. Immunophenotyping involves the binding of specific fluorochrome-conjugated antibodies to specific antigens on the surface of the cells that are being studied (McKinnon, 2019). Fluorochromes such as fluorescein isothiocynate (FITC) and phycoerythrin (PE) are chemicals that possess the ability to absorb the light from the flow cytometer's laser and emits it to a fluorescent light at a higher wavelength (Borowitz, 2014). In the context of cells in the immune system, most common antigens that are targeted in order to define the subpopulation on these cells are T

cells markers (CD3, CD4, CD8) and B cells markers (CD19, CD20) (McKinnon, 2019). In veterinary practice, immunophenotyping and flow cytometry has been used as a tool of refining the diagnosis of lymphoproliferative diseases in cats such as lymphoma (Guzera *et al.*, 2016).

2.4 What are Nutraceuticals?

Nutraceuticals can be defined as food or components of food which are naturally occurring that are postulated to exert positive effects on health (Hayek *et al.,* 2004). Similar to acupuncture and chiropractic therapy, nutraceuticals can be categorized as a form of complementary and alternative medicine (CAM). In a study conducted by Elrod and Hofmeister (2019) which investigates veterinarians' attitudes towards the use of nutraceuticals, a majority of the respondents (51%) would regularly recommend the use of nutraceuticals to their clients. Examples of commonly used nutraceuticals in animal nutrition includes fatty acids, amino acids as well as probiotics and prebiotics (Colitti *et al.*, 2019). According to Elrod and Hofmeister (2019), the most frequently used nutraceutical products are omega-3 fatty acids and glucosamine-based products which are both used in the management of osteoarthritis. In the same study, veterinarians also perceived that nutraceuticals may be beneficial in the treatment of other conditions such as dermatological and gastrointestinal conditions. Apart from treating conditions that are mentioned above, the use of nutraceuticals is also said to be able to modulate the immune system in response against pathogens. Supplementation of fungi-derived beta glucans have been demonstrated to exert positive effects on stimulating the immune system (Akramiene *et al*., 2017). In a study conducted by Haladova *et al.* (2010), dogs that are vaccinated and supplemented with Beta-glucans showed higher antibody titres against rabies compared to dogs that received vaccination only without glucan

supplementation. In contrast, cats that are vaccinated with rabies vaccine and fed with Beta-glucan fortified diets showed no difference in antibody titres compared to the cats in the control group (Byrne *et al.,* 2020). This difference in expected outcome calls for the need to further study the effects of nutraceuticals on each individual species before marketing the product to general consumers. There are a few ways to evaluate the effects of nutraceuticals on the immune system. They include in-vivo studies such as measuring antibody titre response to vaccine as well as measuring circulating lymphocyte concentrations (Hayek *et al.*, 2004). Another nutraceutical product which claims to have immunomodulating properties that is commonly available in Malaysia is N, N-Dimethylglycine (DMG). This product and its supposed immunomodulating properties will be discussed in the next section.

2.5 N, N-Dimethylglycine (DMG) and its Supposed Immunomodulating Properties

N, N - Dimethylglycine (DMG) is a tertiary amino acid and a derivative of the amino acid glycine that is naturally available in low amounts in foods such as grains, legumes and liver (Kern, 2001). DMG is also present endogenously in the animal body as it is synthesized by the liver via the metabolism of choline to glycine in which DMG is an intermediate metabolite (Cupp & Tracy, 2003). Since its discovery in 1943, DMG has been made available to both animals and humans as a form of dietary supplement with most products are claimed to contain 50, 125, or 125 mg of DMG per dosage (Cupp & Tracy, 2003). For its veterinary uses, DMG is often supplemented to athlete animals such as race horses and grey hounds to improve their athletic performances. In a study done by de Oliveira *et al.* (2015), horses that are supplemented with 30 g/day of DMG for 30 days showed an increase in the distance travelled on an inclined treadmill test compared to horses in the control group.

Similarly, greyhounds that are supplemented with DMG also showed a reduction in race time indicating an increase in athletic performance post-supplementation with DMG (Gannon & Kendall, 1982). Another purported benefit of DMG supplementation is the enhancement and modulation of the immune system which includes enhancing both cellular and humoral immune response. Although, it is important to note that the mechanism of action on how DMG modulates the immune system is still unclear. This claim is rather controversial because, as of 2023, there are limited studies conducted on this topic in both animals and humans. Additionally, existing literature regarding this topic seems to contradict one another and fail to reach a consensus. As for the claims of enhancing the humoral immune response, according to a study done on humans by Graber *et al.* (1981), human subjects that were given DMG orally, exhibited four times increase of antibodies after vaccination with pneumococcal vaccine compared to human subjects in the control group. Similarly, in a study done on rabbits, animals that are supplemented with DMG showed four times increase of antibody titre towards influenza antigen post vaccination with a killed influenza virus vaccine (Reap & Lawson, 1990). Interestingly, in contrast, in the only study done on cats, DMG failed to exhibit its immunomodulating capabilities. In the study conducted by Weiss (1992), cats supplemented with DMG showed a lower titre of virus neutralizing antibodies against feline herpesvirus-1 when compared to the control group. Comparably, a study investigating the effects of DMG supplementation on antibody response to influenza vaccine in horses also failed to detect any differences between the antibody titre from horses in the DMG-treated group and the control group (Beech *et al.*, 1987). Findings regarding the effect of DMG supplementation on cellular immune response also varies among different studies. In the human study conducted by Graber *et al.* (1981), lymphocytes that were treated with DMG *in-vitro* showed a significant increase in thymidine uptake post-exposure with mitogens such

as pokeweed, concanavalin A, and phytohemagglutinin which is indicative of increased cellular response post-treatment with DMG. However, in contrast, this effect was not seen in the study done in cats conducted by Weiss (1992), when lymphocytes isolated from the cats' blood were treated with various concentrations of DMG and then exposed to mitogens such as pokeweed, the mean blastogenic response of the lymphocytes did not show a significant difference compared to the untreated lymphocytes. From these few literatures alone, we can observe that no consensus has been reached regarding the immunomodulating properties of DMG.

CHAPTER 3
METHODOLOGY

3.1 Animals and Study Design

This study has acquired the necessary approval from University Putra Malaysia's Institutional Animal Care and Use Committee (IACUC) (U032/2023). The study was conducted at an animal shelter in Semenyih, Selangor and a total of 12 neutered cats of both genders (*male; n = 5, female; n = 7)* from the shelter was recruited for this study. Two weeks before the start of the study, recruited cats was dewormed with a dewormer containing praziquantel, pyrantel pamoate, and fenbendazole (Inovet, Belgium) at a dosage of 1 tab per 10 kg of body weight. For the entirety of the study, all of the cats were conditioned in the same room that they have been in prior to the start of the study. Food in the form of kibbles and water were provided *ad-libitum* throughout the study. Since this study aims to evaluate the immune response of the cats, as recommended by Hofmann-Lehmann et al. (1998), only neutered cats will be included in this study in order to minimize the influence of sex hormones on the results. The cats were randomly assigned to two groups consisting of six cats per group (Group 1 = Control, Group 2 = Treatment). Cats in group 1 were administered with a single dose of *Purevax®Feline 4 Vaccine* (Boehringer Ingelheim, Germany) (Modified Live Rhinotracheitis-Calici-Panleukopenia virus and *Chlamydia psittaci*) only. On the other hand, cats in Group 2 were administered with a single dose of Purevax®*Feline 4* Vaccine (Modified Live Rhinotracheitis-Calici-Panleukopenia virus and *Chlamydia psittaci*) and were orally supplemented with N, N–Dimethylglycine (DMG) in the liquid form (VetriDMG 125mg/ml, VetriSciences, Vermont, USA) at a manufacturer's recommended dosage of 0.5 mL twice a day daily (BID) for the duration of 14 days. All cats were physically examined and temperature was taken prior to vaccination. Monitoring of cats were

done throughout the 14 days of the experiment to ensure no adverse reaction to the vaccination occurred.

3.2 Collection of Samples

Approximately 2 – 3 mL of blood samples from the cats in both groups were collected from either the cephalic vein, saphenous vein, or jugular vein and transferred into 3 mL tubes containing EDTA anticoagulant. Blood from each cat were sampled twice. The first sampling which serves as the baseline data for the experiment took place on day 0, (pre-treatment) at the start of the experiment and prior to vaccination as well as supplementation of DMG. The second sampling took place on day 14 (post-treatment) after 14 days post-vaccination and supplementation of DMG.

3.3 Haematological Analysis

Haematological analysis of the blood samples was carried out within 1-3 hours post sampling. Haematological parameters such as haematocrit (HCT), red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), and platelet (PLT) counts were determined using Celltac Alpha VET MEK-6550K Haematological Analyzer (Nihon Kohden, Japan). Thin blood smear was performed on all blood samples and stained with modified Wright's stain. A differential white blood cell count was carried out on each stained blood smear (100 cells counted per slide) to determine the percentage of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), and basophils (BAS).

3.4 Peripheral Blood Mononuclear Cells (PBMC) Isolation

Peripheral blood mononuclear cells (PBMC)isolation using Histopaque with density of 1.077 g/mL (Sigma Aldrich) was carried out within 24 hours post-sampling. Blood

samples was stored in EDTA tubes at 4 °C prior to PBMC isolation. Briefly, in a 15 mL conical tube, approximately 2 – 3 mL of blood samples were diluted with phosphate buffered saline (PBS) at a ratio of 1:1. The entire volume of the diluted blood was then carefully layered on top a layer of Histopaque of same volume as the blood samples in a new 15 mL conical tube and immediately centrifuged at 1500 rpm for 30 minutes in a swing rotor centrifuge (Kubota Centrifuge, Japan). After centrifuging, the interphase layer (buffy coat) was aspirated out and transferred into a separate 15 mL conical tube and washed with PBS twice. 1X RBC lysis buffer of same volume as blood sample was added to the PBMC pellet to lyse any remaining red blood cells and left to incubate at room temperature for 5 minutes. Next, 5 mL of PBS was added to the RBC lysis buffer-PBMC cell pellet suspension and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the PBMC pellet was resuspended with 5 mL of PBS. A 1:20 dilution of resuspended cells was made by adding 20 µL of resuspended cells to 180 µL of PBS in a 1.5 mL centrifuge tube. From the 1.5 mL centrifuge tube, 10 µL of resuspended cells was mixed with 10 µL of Trypan blue before transferring 10 µL of the suspension into the chamber of haemocytometer for cell counting. After counting, the concentration of the resuspended cells was adjusted to a concentration of 2 x 10^6 cells/mL with PBS.

3.5 Flow Cytometric Analysis of PBMCs

Briefly, 1 mL of resuspended cells with a concentration of 2 x 10^6 cells/mL were transferred into flow cytometer tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended with 1 ml of buffer containing 2% FBS and 2M EDTA. The resuspended cells were once again centrifuged at 1500 rpm for 5 mins. After centrifuging, most of the supernatant was carefully discarded by pipetting to ensure some of the supernatant remains in the flow

cytometer tube. 1 µL of mouse anti-feline CD4-FITC (Southern Biotech, USA) and 1 µL of mouse anti-feline CD8-PE (Southern Biotech, USA) were added to the cells and incubated at 4 °C for 30 minutes. The cells were washed once again with staining buffer containing 2% FBS and EDTA, resuspended in 200 µL of 1% paraformaldehyde, and stored at 4 °C for up to 72h before flow analysis. The percentage of CD4+ and CD8+ T lymphocytes population in the PBMCs were analysed using BD FACSCanto (BD Biosciences, USA). The population of positive cells was determined by gating a similar population of unstained cells. Compensation was also carried out using fluorochrome minus one (FMO) CD4+ and FMO CD8+ controls. Flow cytometry data analysis was carried out using BD FACSDiva software (BD Biosciences, USA) and data was acquired for 50,000 events.

3.6 Statistical Analysis

All data from the treatment group and control group are presented as median (interquartile range, IQR). Statistical analysis was done on GraphPad Prism 9 software (GraphPad Software Inc., USA) to evaluate significant differences within groups at different time points and significant differences between groups at different time points. Data was analysed using Wilcoxon Matched Pairs Test to determine significant difference within groups at Day 0 versus Day 14 and Mann-Whittney Test to determine significant difference between groups at Day 14. $P \leq 0.05$ was considered statistically significant.

CHAPTER 4
RESULTS AND FINDINGS

4.1 Haematological Parameters and Differential White Blood Cell Percentage

All results and statistical analyses of differences between control group and treatment group pertaining to haematological parameters (RBC, HCT, HGB, PLT and WBC), and differential white blood cell percentage (NEU, LYM, MON, EOS, and BAS) are presented in Figure 1 and Figure 2 respectively. As shown in Figure 1**,** generally, at Day 14 of the trial (post-treatment), values for parameters such as RBC, HCT, HGB, PLT, and WBC did not differ significantly ($p \geq 0.05$) between control group and treatment group. From day 0 to day 14, both groups exhibited a decreasing trend in values for parameters such RBC, HCT, HGB, and PLT. Additionally, from day 0 to day 14, both groups exhibited an increasing trend for WBC. However, both of the decreasing and increasing trend were not statistically significant ($p \geq 0.05$).

It is also important to note that as shown in Figure 1, most of the values for haematological parameters of both group for both day 0 and day 14 are within the normal reference range or slightly above the upper normal limits [RBC value for treatment group on day 0; 10.50 x 10^{12}/L (10.83 – 9.49) (Appendix 1), and HCT value for treatment group on day 0; 45.5% (47.53 – 41.13)] (Appendix 1). The values for PLT for both groups on both day 0 and day 14, are well below the reference range.

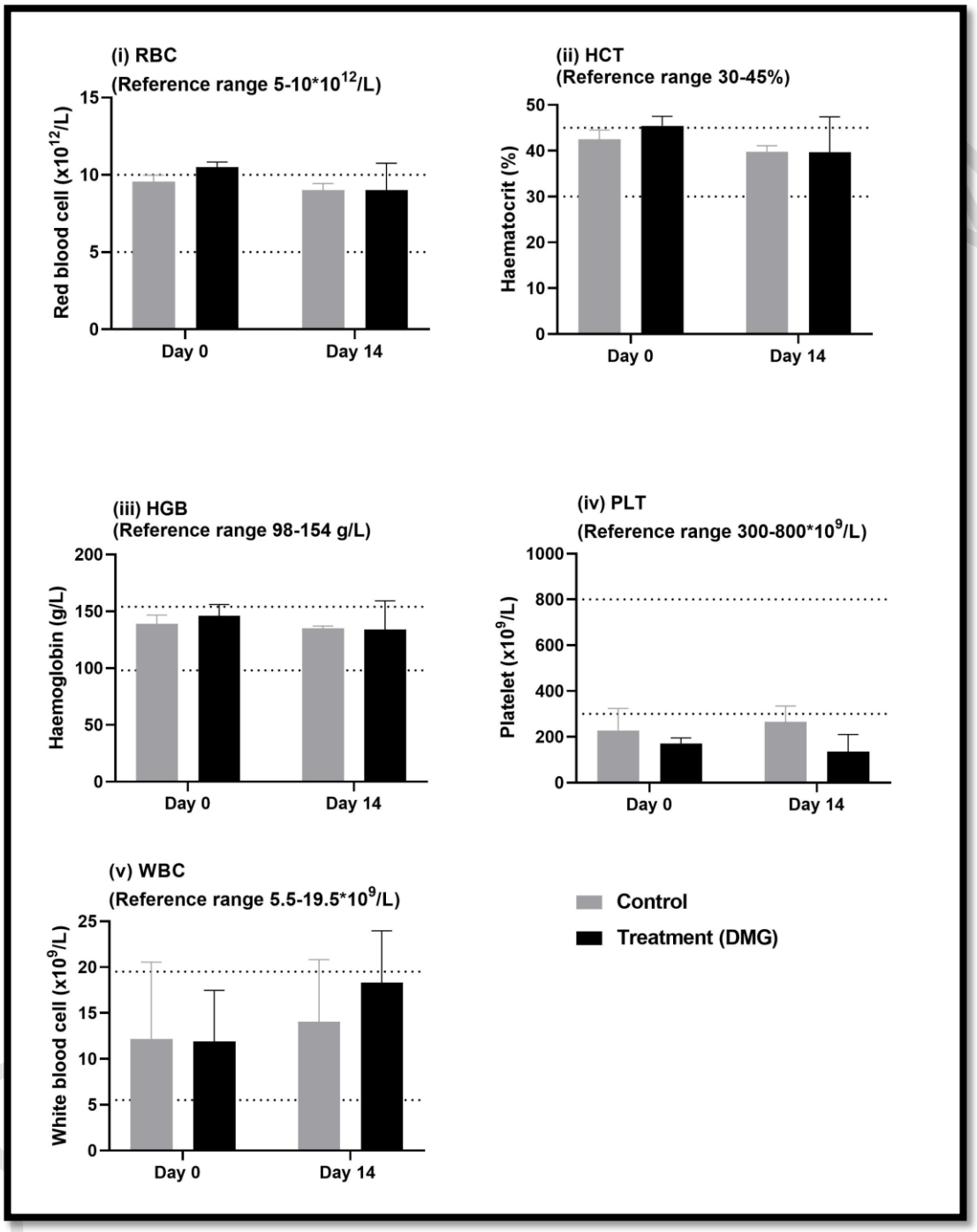

Figure 1 Effect of N, N-Dimethylglycine Supplementation on Haematological Parameters. Data was analysed using Wilcoxon Matched Pairs Test to determine significant difference within Groups at Day 0 versus Day 14 and Mann-Whittney Test to determine significant difference between Group at Day 14. $p \leq 0.05$ is considered to be statistically significant. Data is presented as median (IQR). Reference range is based on feline data adapted in part from multiple sources, including Latimer KS, *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology*, 5th ed., Wiley-Blackwell, 2011; and Weiss DJ, Wardrop KJ, *Schalm's Veterinary Hematology*, 6th ed., Wiley-Blackwell, 2010.

As for the findings for differential white blood cell percentage (Figure 2), generally, at day 14 of the trial (post-treatment), values for parameters such as MON, EOS, and BAS did not differ significantly ($p \geq 0.05$) between control group and treatment group. Interestingly, at day 14 of the trial, cats in the treatment group exhibited significantly lower NEU percentage compared to the control group [60.00% (64.25 – 57.75) vs 67.50% (71.25 – 63.25); $p \leq 0.05$] (Appendix 2). Conversely, at day 14 of the trial, cats in the treatment group exhibited significantly higher LYM percentage compared to the control group [33.50% (36.50 – 31.50) vs 26.50% (28.50 – 22.00); $p \leq 0.05$] (Appendix 2)

Lastly as shown in Figure 2 with the exception of LYM percentage of the control group on Day 0 [26.50% (28.50 – 22.00)] (Appendix 2), all of the values for differential white blood cell percentage parameters of both group for both day 0 and day 14 are within the normal reference range or slightly above the upper normal limit.

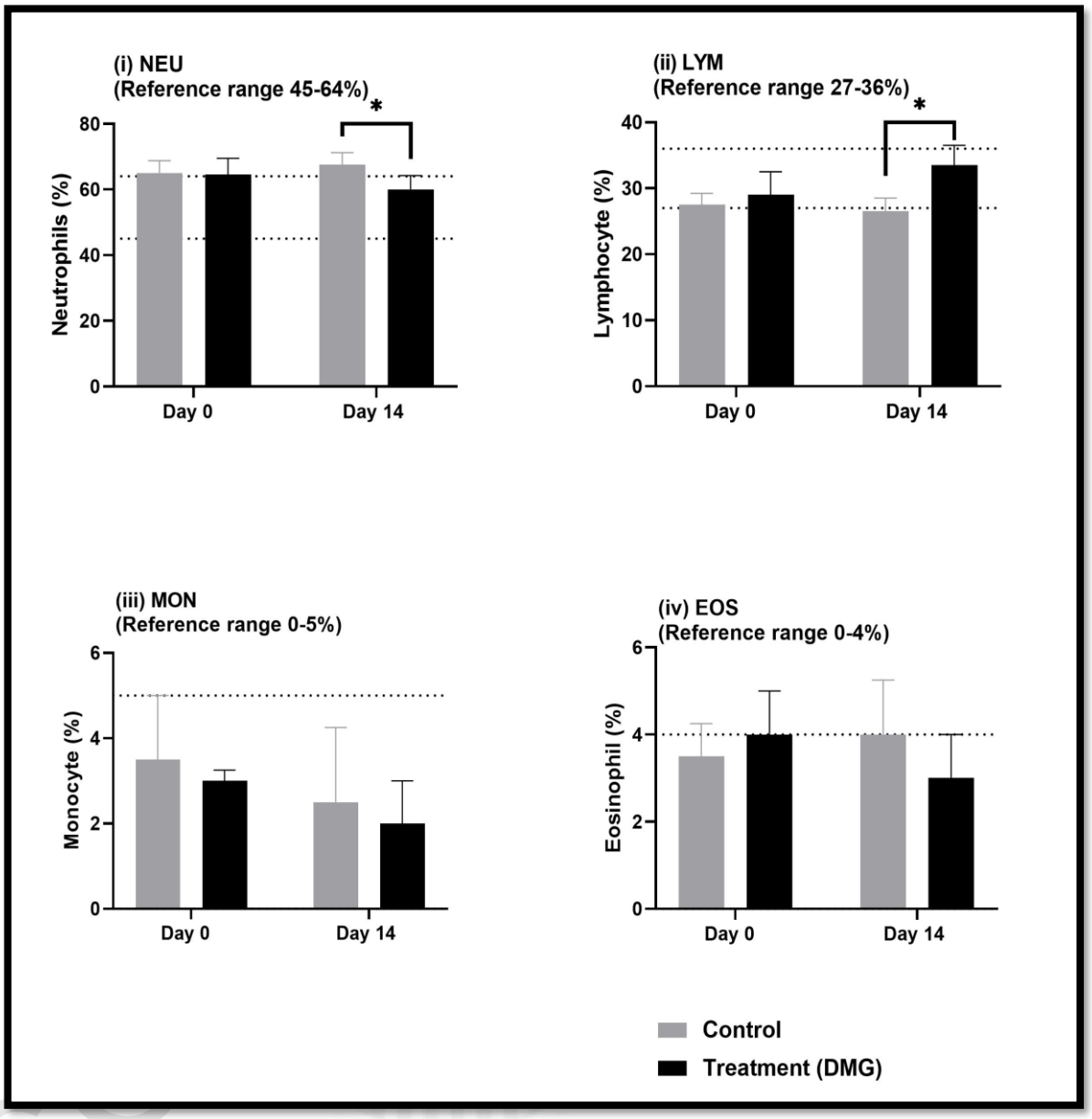

Figure 2 Effect of N, N-Dimethylglycine Supplementation on Differential White Blood Cell Percentage. Data was analysed using Wilcoxon Matched Pairs Test to determine significant difference within Groups at Day 0 versus Day 14 and Mann-Whittney Test to determine significant difference between Group at Day 14. $p \leq 0.05$ is considered to be statistically significant, depicted with * symbol. Data is presented as median (IQR). Reference range is based on feline data adapted in part from multiple sources, including Latimer KS, *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology*, 5th ed., Wiley-Blackwell, 2011; and Weiss DJ, Wardrop KJ, *Schalm's Veterinary Hematology*, 6th ed., Wiley-Blackwell, 2010.

4.2 Lymphocyte Subsets (CD4+, CD8+ T cell)

All results and statistical analyses of differences between control group and treatment group pertaining to lymphocyte subsets (CD4+ T cell percentage, CD8+ T cell percentage, and CD4+ to CD8+ ratio) is presented in Figure 3 respectively. As stated in Figure 3**,** cats in the control group exhibited a significant increase in CD4+ T cell percentage from day 0 to day 14 [14.45% (22.53 – 10.38) vs 28.35% (36.08 – 22.90); $p \leq 0.05$] (Appendix 3). In contrast, cats in the treatment group showed a non-significant decrease in CD4+ T cell percentage from Day 0 to Day 14. Due to this, at day 14 (post-treatment) the control group had a significantly higher CD4+ T cell percentage compared to the treatment group [28.35% (36.08 – 22.90) vs 6.15% (10.18 – 3.70); $p \leq 0.05$] (Appendix 3). As for CD8+ T cell percentage, both groups exhibited a non-significant ($p \geq 0.05$) decreasing trend from day 0 to day 14, although it seems that the decrease in CD8+ T cell percentage was prominent in the treatment group. Lastly, the control group showed a slight increase, albeit non-significant ($p \geq 0.05$ of CD4:CD8 ratio from day 0 to day 14. Conversely, the treatment group exhibited a non-significant ($p \geq 0.05$) reduction of CD4:CD8 ratio from Day 0 to Day 14. It is important to note that as seen in Figure 3**,** the CD4:CD8 ratio of both groups in both day 0 and day 14 were within the normal range.

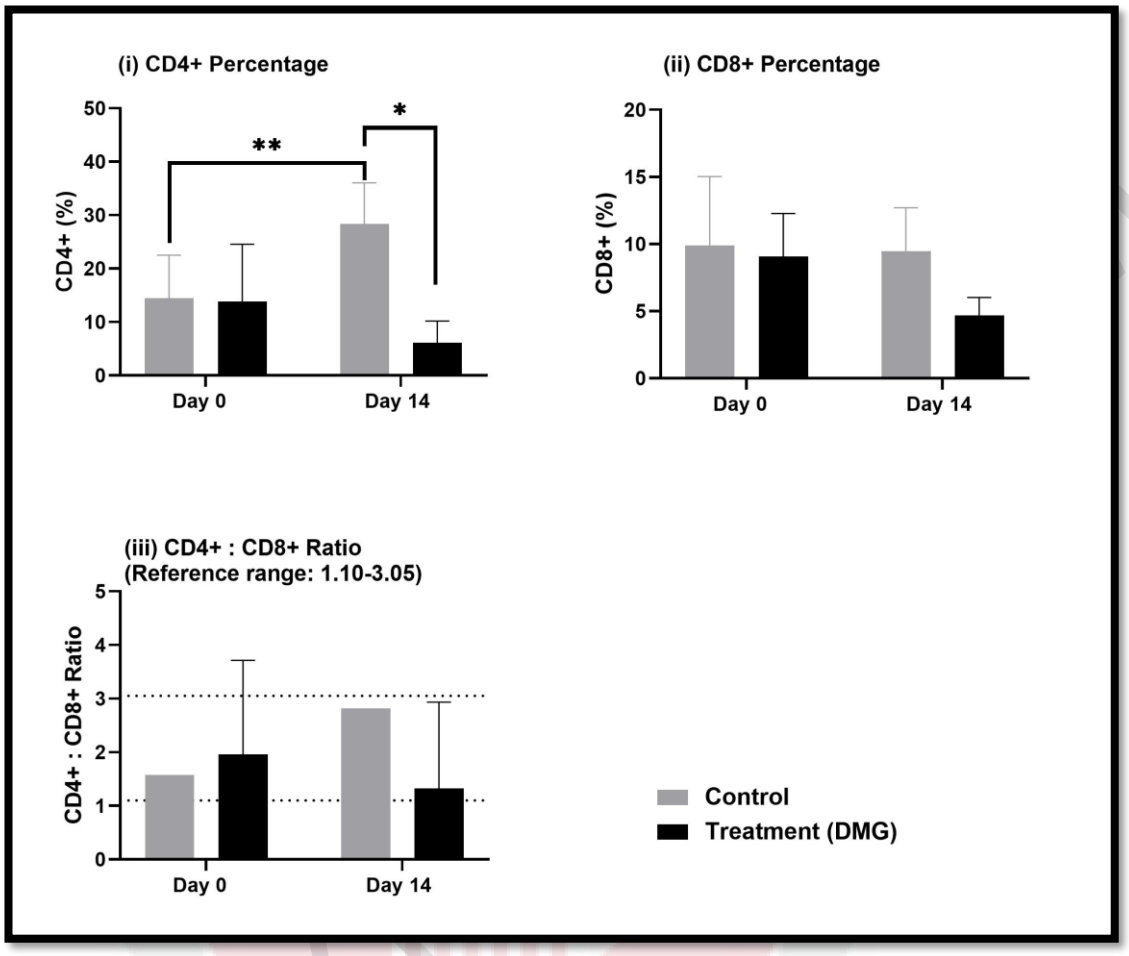

Figure 3 Effect of N, N-Dimethylglycine Supplementation on Lymphocyte Subsets (CD4+, CD8+ T cell). Data was analysed using Wilcoxon Matched Pairs Test to determine significant difference within Groups at Day 0 versus Day 14 (significant difference depicted by ** symbol) and Mann-Whittney Test to determine significant difference between Group at Day 14 (significant difference depicted by * symbol). $p \leq 0.05$ is considered to be statistically significant. Data is presented as median (IQR). Reference range is based on data from Byrne, K. M., Kim, H. W., Chew, B. P., Reinhart, G. A., & Hayek, M. G. (2000). A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. *Veterinary immunology and immunopathology*, *73*(2), 167–182. https://doi.org/10.1016/s0165-2427(99)00163-4

CHAPTER 5
DISCUSSION

The impacts of oral supplementation of various nutraceuticals on immune response towards infectious diseases and their influence on vaccine response are still being studied in various animal species (Filho *et al.*, 2019; Mayer *et al.,* 2019; Mohamed *et al.,* 2019). In the context of DMG, studies pertaining to the relationship between DMG supplementation and its effects on immune response till this day is still relatively scarce and infinitesimal, especially for the feline species. As noted earlier, the only study that investigated the effects of DMG supplementation on the immune response of cats was conducted back in the early 90s by Weiss (1992). As a result of this, further explanations regarding the DMG's mechanism in affecting the feline immune system can only be extrapolated from earlier research conducted on other species such as poultry (Kalmar *et al.,* 2012), and model of human keratinocyte (Lendvai *et al.,* 2023). In this discussion, based on the experimental results and findings, focus will be made on the effects of DMG supplementation on haematological (complete blood count) parameters, differential white blood cell percentage, and lymphocyte subset.

Based on the experimental results and findings, the results showed that both cats in the control group and treatment group showed no significant changes from pre-treatment (day 0) to post-treatment (day 14) for parameters pertaining to the complete blood count (Table 1**)** such as RBC, HCT, HGB, PLT, and WBC. Additionally, the values for RBC, HCT, HGB, and WBC of both groups are also within the normal range or slightly above the upper normal limit [RBC value for treatment group on Day 0; 10.50 x 10^{12}/L (10.83 – 9.49), and HCT value for treatment group on Day 0; 45.5% (47.53 – 41.13)] (Figure 1) (Appendix 1) for both pre-treatment and post-treatment. The slightly high RBC value for treatment group on day 0 and HCT value for treatment

group on Day 0 were most probably due to haemoconcentration due to the cats being slightly dehydrated prior to the start of the study. This can be proven as the RBC and HCT values normalised on day 14 due to the constant checking and refilling of the water bowl throughout the study which ensured that the cats remain hydrated throughout the study. This is unsurprising as the cats were from an animal shelter, hence the cats' nutrition and hydration would probably not be optimal. Considering these findings, we can extrapolate that short term supplementation of DMG is safe for the cats and does not affect the normal values of haematological parameters. This finding supports the claims of the manufacturer, whereby it is claimed that DMG is not toxic and does not cause any side effects. Clinically, throughout the entire study, cats that received daily bi-supplementation of DMG also did not exhibit any abnormal clinical signs and behaviours. Indeed, supplementation of DMG is also proven to be safe when consumed in large doses and for long term duration. For instance, chickens that were supplemented with DMG in the form of dimethylglycine sodium salt (Na-DMG) at a dosage of 10 g/kg did not induce any toxicity or impaired their broiler performances parameters (Kalmar et al., 2012). As for the PLT values (Figure 1**)**, both groups showed lower PLT values compared to the reference range on both day 0 and day 14. One possible explanation for this is due to the long interval between sample collection and sample processing. According to Hardy *et al.* (2020), a significant decrease in apparent platelet count was observed over time from the blood draw, both statistically ($p < 0.001$) and clinically.

Moving on to differential white blood cell percentage, our results (Figure 2) showed that for parameters such as BAS, EOS, and MON percentage, there was no significant difference ($p \geq 0.05$) in both groups for both day 0 and day 14. Moreover, the percentage of BAS, EOS, and MON of both groups were also within the normal reference range. However, on day 14, the treatment group had significantly lower

NEU percentage [60.00% (64.25 – 57.75) vs 67.50% (71.25 – 63.25); p ≤ 0.05] (Appendix 2) and significantly higher LYM percentage [33.50% (36.50 – 31.50) vs 26.50% (28.50 – 22.00); p ≤ 0.05] (Appendix 2) compared to the control group. There are a few possible explanations on the lower NEU percentage of the treatmentl group. Firstly, the decrease in neutrophil in the treatment group may signify DMG's ability to reduce the overall inflammatory process that is ongoing in the cats. It is common knowledge that an increase in neutrophil counts may signify an ongoing inflammatory process. As mentioned by Lendvai *et al*. (2023), when studied in models of human keratinocytes, mimicking inflammatory disease such as contact dermatitis, dimethylglycine exerts robust anti-inflammatory as well as antioxidant properties. Other than that, the reduction in neutrophil may also signify the anti-inflammatory properties of bromelain as the DMG supplement that was used in the study contained bromelain at a concentration of 2 mg/mL. Bromelain is an enzyme extracted from pineapples and considered to be an alternative to non-steroidal anti-inflammatory drugs. Bromelain has shown to have anti-inflammatory properties in various in-vivo and in-vitro studies (Pavan *et al.,* 2012). As for the significantly higher lymphocyte percentage in the treatment group, this can be attributed to the synergistic effects of both of the vaccine and DMG. In several studies done in the past, DMG has shown to have lymphoproliferative properties when studied in both *in-vitro* and *in-vivo* (Graber *et al*., 1981; Reap & Lawson, 1990). Unfortunately, DMG's mechanism in inducing lymphoproliferation as well as its effects in modulating the immune system is not fully elucidated. One of the proposed mechanisms is via inhibition of T-suppressor cells as well as the enhancement of T-cell presentation of the OKT4 antigen (Cupp & Tracy, 2003). Additionally, the increase of lymphocyte in the treatment group can also be due to the effect of the vaccine as according to Vojtek *et al.* (2021), vaccines also exhibit lymphoproliferative effects. Interestingly, it is unclear

why the vaccine did not show the same effect in the control group. Possible explanations include malnutrition as well as individual variation between the subjects. Since the cats are from a high-density animal shelter, non-optimal nutritional intake is to be expected. In cats, states of malnourishment can lead to an impaired immune response to vaccine (Hartmann *et al.,* 2022).

Regarding the impact of DMG supplementation on lymphocyte subset (Figure 3), our findings indicates that DMG appears to have no significant impact in influencing CD4+ to CD8+ ratio. Based on our results, cats of both groups demonstrated CD4+ to CD8+ ratio values that is within the reference range of 1.10 to 3.05 (Byrne *et al.,* 2000). As for the percentage of CD4+ T cell, control group demonstrated a significant increase in CD4+ percentage from day 0 to day 14 [14.45% (22.53 – 10.38) vs 28.35% (36.08 – 22.90); $p \leq 0.05$] (Appendix 3). This is most probably attributed to the immune response towards the vaccine that was administered back in day 0. Notably, conversely, from day 0 to day 14, the treatment group demonstrated a non-significant reduction ($p \geq 0.05$) of both CD4+ and CD8+ percentage. We speculate that DMG might cause other lymphocyte subsets (B cells, natural killer cells) to increase and alter balance of lymphocyte subset. However, these parameters were not measured in the current study. The reduction in CD4+ after the supplementation of DMG supplement might also be due to effects of bromelain in the DMG supplementation. According to Secor *et al.* (2009), in-vitro treatment of lymphocytes with bromelain caused a reduction in the activation of CD4+ T cells and lowers the expression of CD25. Hence, based on our results, we can extrapolate that supplementation of DMG does not have a significant effect on lymphocyte subset particularly CD4+ and CD8+ T cell. Therefore, more comprehensive study must be conducted in the future to fully understand DMG's immunomodulatory properties in the feline species.

CHAPTER 6
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, the present study investigated the effects of DMG supplementation on hematological parameters and the percentage of CD4+ and CD8+ T cells in cats following core vaccination. Cats supplemented with DMG displayed lower neutrophils and higher lymphocytes, suggesting potential anti-inflammatory and lymphoproliferative effects. However, DMG did not significantly influence the percentages of CD4+ and CD8+ T cells. Further research is warranted to investigate the effects of DMG on other lymphocyte subsets, such as B cells and natural killer cells, and to explore the long-term effects of DMG supplementation on feline immune health.

6.2 Limitations

The present study has several limitations. First, the study only evaluated the effects of DMG supplementation for a short period of 14 days. It is possible that longer-term supplementation may be required to observe a more pronounced effect of the immune system parameters. Second, the usage of cats from an animal shelter most probably resulted in high variability between subjects due to unknown health history. This high variability between subjects will lead to more unknown confounding factors which may have affected our data. Lastly, the current study only evaluated the percentage of CD4+ and CD8+ T cell only. This data provides limited information on the cat's immune function. Hence, based on the limitations of the study. Several recommendations should be taken into account for future study.

6.3 Recommendations

To further elucidate the potential benefits of DMG supplementation on feline immune function, future research should address the limitations of the present study. First, evaluating the effects of DMG supplementation over an extended period would allow for a more comprehensive assessment of its potential long-term effects on the feline immune system. In addition, investigating the functional effects of DMG supplementation, such as antibody production and lymphocyte proliferation, would provide a more direct and comprehensive measure of its impact on immune function. Additionally, examining the effects of DMG supplementation on other lymphocyte subsets, such as B cells and natural killer cells, would broaden our understanding of its immunomodulatory properties. Finally, the use of specific pathogen-free (SPF) cats and controlled animal research facility in future studies would eliminate confounding factors arising from pre-existing infections or environmental stressors, ensuring that the observed effects are attributable solely to DMG supplementation.

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APPENDICES

i. Client Consent Form

Consent Form

Department of Department of Veterinary Pathology & Microbiology
Faculty of Veterinary Medicine
Universiti Putra Malaysia

Title of Study :

Effect of N, N-Dimethylglycine supplementation on Haematological Parameters and Frequency of CD4+ and CD8+ T Cells in Cats Post-Vaccination

AUP approval no : (IACUC) (U032/2023)

Period of Study : 30 July 2023 – 18 September 2023 (7 weeks)

Location :

At the participating shelter. Sample analysis will be done in Faculty of Veterinary Medicine, UPM

Purpose of the Study :

1. To examine the effects of N, N-Dimethylglycine (DMG) supplementation on haematological parameters in cats post-vaccination
2. To determine the frequency of CD4+ and CD8+ T Cells from Peripheral Blood Mononuclear Cells (PBMC) post supplementation with DMG and in cats post-vaccination

Involvement :

1. 12 neutered cats from participating shelter.
2. Dr. Farina Mustaffa Kamal (Senior Lecturer in Veterinary Immunology)
3. Syahir Aiman Bin Shahril Agus (4th year DVM student)

Procedures :

1. 12 neutered male cats will be examined and dewormed prior to the start of the study. The cats will also be kept in the same environment away from non-recruited cats for 1 month (28 days) to allow physiological acclimatization.

2. Recruited cats will be vaccinated with a single dose of Purevax Feline 4 Vaccine.

3. Recruited cats will be supplemented with 0.5 mL of VetriDMG immune booster or placebo (normal saline) twice a day for 14 days.

4. Blood sampling of recruited cats will be done twice. Once before administration of vaccine and once after administration of vaccine (Day 14).

Possible Risk(s) **:** No adverse or negative side effect is to be expected with the supplementation of VetriDMG. Even though vaccination is considered safe, according to Tizard (2021), previous data shows that 51.6 adverse events occurred per 10 000 cats vaccinated. Some cats might experience mild-side effects such as; local swelling at the vaccination site, decreased activity and appetite. These are expected to last 1 – 2 days at most. If cats are seen to exhibit signs of these side-effects for more than two days, the cats will be removed from the study. Lastly, if a cat experiences severe side effects such as hives, dyspnoea and diarrhoea, this warrants the immediate removal of affected animal from the study.

Tizard I. R. (2021). Feline vaccines. Vaccines for Veterinarians, 167–178.e1. https://doi.org/10.1016/B978-0-323-68299-2.00023-X

Confidentiality **:** Confidential

Voluntary Participation **:** Voluntary

Financial Compensation **:** N/A

Declaration:

I have read and understand the above explanation in regard to procedures listed above and the possible risk(s) associated with vaccination and supplementation of VetriDMG. I understand that my cat(s) may be withdrawn from the study at any time based on the veterinarian's judgement. I also understand that the data collected from my cat(s) may be used for academic and presentation purposes. I have had the opportunity to ask questions and clarify any concerns I may have had, and I am satisfied with the answers provided. I freely and voluntarily give my consent for my cat(s) to participate in this study

Name of Owner: _____ Signature: _____

Contactno/Email: _____ Date: _____

Signature: _____

Project Conductor: Syahir Aiman Bin Shahril Agus

Contact no/Email: 013-610453/ 200749@student.upm.edu.my Date : _____

Principal Investigator: Dr. Farina Mustaffa Kamal Signature: _____

Contact no/Email: 603 8609 3466/ farina@upm.edu.my Date : _____

For further enquiries or concerns, do contact:

1. Principal Investigator:
   Dr. Farina Mustaffa Kamal, Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 UPM Serdang, Selangor
   Email: farina@upm.edu.my Contact No.: +60386093466
2. Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor. Email: iacuc@upm.edu.my or contact no: +603-97691244/1605

ii. Animal Monitoring Sheet

Effect of N, N-Dimethylglycine supplementation (DMG) on Haematological Parameters and Frequency of CD4+ and CD8+ T Cells in Cats Post-Vaccination

Animal Monitoring Sheet

PI/Student: Dr. Farina Mustaffa Kamal/Syahir Aiman Shahril Agus IACUC Approval no.:

Date of Experimentation: Animal ID:

Procedure: Post-vaccination monitoring, feeding trials monitoring, blood withdrawal

DATE / OBSERVATION	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
ACTIVITY Normal =0 Isolated =1 Inactive= 2 Moribund = 3							
APPETITE AND WATER INTAKE Normal= 0 Decreased= 1							
VACCINATION SITE Normal = 0 Slight swelling = 1 Large swelling = 2 Bleeding = 3							
URINATION Positive = 0 Negative = 1							
DEFECATION +ve/-ve (Fecal Score) 1-5 (Liquid feces – hard, crumbly feces)							
TPR T: 38.0 -39.1 P: 140-220 R: 25-40							
OTHER COMMENTS							
MONITORED BY							

Effect of N, N-Dimethylglycine supplementation (DMG) on Haematological Parameters and Frequency of CD4+ and CD8+ T Cells in Cats Post-Vaccination

Animal Monitoring Sheet

PI/Student: Dr. Farina Mustaffa Kamal/Syahir Aiman Shahril Agus IACUC Approval no.:

Date of Experimentation: Animal ID:

Procedure: Post-vaccination monitoring, feeding trials monitoring, blood withdrawal

DATE / OBSERVATION	DAY 8	DAY 9	DAY 10	DAY 11	DAY 12	DAY 13	DAY 14
ACTIVITY Normal =0 Isolated =1 Inactive= 2 Moribund = 3							
APPETITE AND WATER INTAKE Normal= 0 Decreased= 1							
VACCINATION SITE Normal = 0 Slight swelling = 1 Large swelling = 2 Bleeding = 3							
URINATION Positive = 0 Negative = 1							
DEFECATION +ve/-ve (Fecal Score) 1-5 (Liquid feces – hard, crumbly feces)							
TPR T: 38.0 -39.1 P: 140-220 R: 25-40							
OTHER COMMENTS							
MONITORED BY							

iii. Experimental Data and Results

Appendix 1 Effect of N, N-Dimethylglycine Supplementation on Haematological Parameters.

Parameters	Days	Control Group (n=6)	Treatment Group (n=6)
Red blood cells (RBC) (10^{12}/L)	Day 0	9.56 (9.99 – 8.62)	10.50 (10.83 – 9.49)
	Day 14	9.02 (9.45 – 8.64)	9.01 (10.75 – 8.27)
Haematocrit (HCT) (%)	Day 0	42.5 (44.5 – 40.1)	45.5 (47.53 – 41.13)
	Day 14	39.80 (41.10 – 39.25)	39.70 (47.40 – 34.73)
Haemoglobin (HGB) (g/dL)	Day 0	139.00 (146.75 – 133.75)	145.00 (156.00 – 131.25)
	Day 14	135.00 (137.00 – 131.75)	134.00 (159.25 – 117.00)
Platelet (PLT) (10^9/L)	Day 0	226.00 (323.50 – 184.00)	171.50 (195.50 – 96.00)
	Day 14	265.00 (334.50 – 207.50)	135.50 (210.25 – 122.25)
White blood cells (WBC) (10^9/L)	Day 0	12.150 (20.55 – 10.45)	11.90 (17.48 – 10.65)
	Day 14	14.05 (20.83 – 9.30)	18.30 (23.98 – 14.68)
*Data is presented as median (IQR) a Statistically significant difference between D0 and D14 b Statistically significant difference between Groups at D14			

Appendix 2 Effect of N, N-Dimethylglycine Supplementation on Differential White Blood Cell Percentage.

Parameters	Days	Control Group (n=6)	Treatment Group (n=6)
Neutrophil (NEU) (%)	Day 0	65.00 (68.75 – 63.25)	64.50 (69.50 – 61.25)
	Day 14	67.50 (71.25 – 63.25) [b]	60.00 (64.25 – 57.75) [b]
Lymphocyte (LYM) (%)	Day 0	27.50 (29.25 – 24.50)	29.00 (32.50 – 22.50)
	Day 14	26.50 (28.50 – 22.00) [b]	33.50 (36.50 – 31.50) [b]
Monocyte (MON) (%)	Day 0	3.50 (5.00 – 2.75)	3.00 (3.25 – 2.00)
	Day 14	2.50 (4.25 – 1.75)	2.00 (3.00 – 2.00)
Eosinophil (EOS) (%)	Day 0	3.50 (4.25 – 2.75)	4.00 (5.00 – 3.00)
	Day 14	4.00 (5.25 – 2.75)	3.00 (4.00 – 2.00)
Basophil (BAS) (%)	Day 0	0.00 (1.00 – 0.00)	0.00 (0.25 – 0.00)
	Day 14	0.00 (0.25 – 0.00)	0.00 (0.25 – 0.00)

*Data is presented as median (IQR)
a Statistically significant difference between D0 and D14
b Statistically significant difference between Groups at D14

Appendix 3 Effect of N, N-Dimethylglycine Supplementation on Lymphocyte Subsets (CD4+, CD8+ T cell).

Parameters	Days	Control Group (n=6)	Treatment Group (n=6)
CD4+ T Cell (CD4) (%)	Day 0	14.45 (22.53 – 10.38) [a]	13.85 (24.55 – 3.53)
	Day 14	28.35 (36.08 – 22.90) [ab]	6.15 (10.18 – 3.70) [b]
CD8+ T Cell (CD8) (%)	Day 0	9.90 (15.09 – 4.95)	9.05 (12.28 – 5.75)
	Day 14	9.45 (12.70 – 3.70)	4.70 (6.03 – 3.18)
CD4:CD8 Ratio	Day 0	1.58 (5.25 – 0.74)	1.96 (3.71 – 0.28)
	Day 14	2.82 (14.01 – 2.00)	1.32 (2.94 – 1.06)

*Data is presented as median (IQR)
a Statistically significant difference between D0 and D14
b Statistically significant difference between Groups at D14