MICROBIAL, IMMUNOLOGICAL AND HISTOPATHOLOGICAL STUDIES ON THE EFFECTS OF PROBIOTIC AND *Phaleria macrocarpa* LEAVES EXTRACT IN IMMUNOCOMPROMISED NEW ZEALAND WHITE RABBITS

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ABSTRACT

The purpose of this study is to investigate the advantages of using a novel formulated probiotic and *Phaleria macrocarpa* leaves extract on immunocompromised New Zealand White (NZW) rabbits. Forty rabbits were divided into four groups; three group were induced with immunosuppressant drug (Azathioprine, at 500 mg/kg/day) and the forth group served as control. The first group of immunocompromised rabbits treated with the formulated probiotic, the second group with *P. macrocarpa* extract, while the third group without any treatment. The results were assessed through two different period of time; induction period (14 days) and treatment period (14 days). Results showed that Feed Conversion Ratio (FCR) of immunocompromised groups were decreased significantly ($P \leq 0.05$) during the induction period; -2.07 for the non-treated, -1.06 for treated with the probiotic, and -2.54 for the treated with *P. macrocarpa* extract, compared to control group (1.34). However, during the treatment period, the FCR continued decreased (insignificantly) within the non-treated group (as -8.99), but increased significantly in both probiotic treated group (0.70), and extract treated group (0.66), compared to control (1.49). The microbiological and immunological findings indicated that treatment with *P. macrocarpa* extract enhanced the rabbits to maintain significantly the full blood cells count ($9.16 \pm 0.39 \times 10^9$) closer to normal levels of control group ($9.68 \pm 0.30 \times 10^9$), in comparison to probiotic treated group ($5.42 \pm 2.20 \times 10^9$) and non-treated group ($3.20 \pm 0.59 \times 10^9$). The percentage of lymphocytes were decreased significantly in the extract treated group (32%), probiotic treated (25%), and non-treated (20%), compared to control group (66%). The histopathological findings declared critical changes occurred to the colon tissue of non-treated rabbits, including loss of regular mucosal folds, variable degrees of mucosal edema and congestion, submucosal hemorrhage, accumulative score of colon gross anatomy, and substantial serious lesions. Meanwhile, most of these histopathological changes were highly maintained in treated rabbits, either with *P. macrocarpa* extract or with the formulated probiotic. As a conclusion, the daily uptake of *P. macrocarpa* extract or the formulated probiotic could improve significantly (both specific and nonspecific) immune responses of immunocompromised rabbits, and maintain the damaged colon tissue (caused due to the immunosuppressant drug). The study implicates that the formulated probiotic and *P. macrocarpa* extract products can serve as supplements in reducing medical complications of domesticated animals or human patients under certain condition.

KAJIAN MIKROB, IMUNOLOGI DAN HISTOPATOLOGI MENGENAI KESAN PROBIOTIK DAN EKSTRAK TUMBUHAN *Phaleria macrocarpa ARNAB* DIIMMUNOKOMPROMI

ABSTRAK

Kajian ini bertujuan mengkaji kebaikan menggunakan formulasi ekstrak daun probiotik dan *Phaleria macrocarpa* ke atas arnab putih New Zealand yang diimunokompromi. Empat puluh ekor arnab telah digunakan, terbahagi kepada empat kumpulan; tiga dircetuskan dengan ubat tahan imun (Azathioprine, pada 500 mg/kg/hari), dan yang keempat berfungsi sebagai kawalan sihat. Kumpulan pertama arnab yang diimunokompromi dirawat dengan rumusan probiotik, yang kedua dirawat dengan ekstrak *P. macrocarpa*, sementara yang ketiga dibiarkan sembuh tanpa sebarang rawatan. Keputusannya menunjukkan bahawa Nisbah Pertukaran Makanan (NPM) kumpulan yang diimunokompromi telah menurun secara signifikan ($P \leq 0.05$) dalam tempoh masa induksi; -2.07 untuk yang tidak dirawat, -1.06 yang dirawat dengan probiotik, dan -2.54 untuk arnab yang dirawat dengan ekstrak *P. macrocarpa*, berbanding dengan kumpulan kawalan (1.34). Walau bagaimanapun, dalam tempoh rawatan, NPM terus menurun (secara tidak signifikan) untuk kumpulan yang tidak dirawat (-8.99), tetapi meningkat secara signifikan dalam kedua-dua kumpulan yang menerima rawatan probiotik (0.70), dan kumpulan yang menerima rawatan ekstrak (0.66), berbanding dengan kumpulan kawalan (1.49). Dapatan mikrobiologi dan immunologi menunjukkan bahawa rawatan dengan ekstrak *P. macrocarpa* meningkatkan serta mengekalkan secara signifikan kiraan sel darah penuh ($9.16 \pm 0.39 \times 10^9$) lebih hampir dengan aras normal kumpulan kawalan($9.68 \pm 0.30 \times 10^9$), berbanding dengan kumpulan yang dirawat dengan probiotik ($5.42 \pm 2.20 \times 10^9$) dan kumpulan yang tidak dirawat ($3.20 \pm 0.59 \times 10^9$). Peratusan limfosit turut menurun secara signifikan dalam kumpulan yang menerima rawatan ekstrak (32%), rawatan probiotik (25%), dan bukan dirawat (20%), berbanding dengan kumpulan kawalan (66%). Dapatan hispatologi mengesahkan berlakunya perubahan kritikal kepada tisu kolon arnab tidak dirawat, termasuk kehilangan lipatan mukosa biasa, pelbagai darjah edema mukosa dan kesesakan, pendarahan submukosa, skor kasar terkumpul anatomi kolon, dan luka yang serius. Sementara itu, kebanyakan perubahan hispatologi dikekalkan dalam arnab yang dirawat, sama ada dengan ekstrak *P. macrocarpa* atau dengan probiotik rumusan. Kesimpulannya, pengambilan harian estrak *P. macrocarpa* atau probiotik rumusan boleh meningkatkan secara signifikan (spesifik dan tidak spesifik) respon imun arnab yang diimunokompromi, dan mengekalkan tisu kolon yang rosak (disebabkan oleh ubat tahan imun). Kesimpulan daripada kajian menunjukkan bahawa probiotik rumusan dan ekstrak *P. macrocarpa* boleh dijadikan asas suplemen dalam mengurangkan komplikasi perubatan yang lazimnya diberikan kepada haiwan peliharaan atau pesakit yang berada dalam keadaan tertentu.

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Alcian Blue - Periodic Acid Schiff	Alcian Blue-PAS
Analytical Profile Index	API
Antigen Presenting Cells	APC_S
B cell Activation Factor	BAFF
Blood Agar	BA
Bursa cell	B cell
Cluster of Differentiation	CD
Colony Forming Units	CFU
Compete Blood Count	CBC
de MAN, ROGOSA and SHARPE	MRS broth
Degree Celsius	°C
Dendritic Cell	DC
Eosin Methylene Blue	EMB
Epigallocatechin-3-gallate	EGCG
Feed Conversion Ratio	FCR
Feed Efficiency Ratio	FER
Feed Intake	FI
Food and Agriculture Organization	FAO
Fragment Crystallizable Receptor	Fc receptor
Full Blood Count	FBC
Gastrointestinal Tract	GIT
Gram	g
Gut Associated Lymphoid Tissue	GALT
Hematoxylin and Eosin	H&E
Immunoglobulin	Ig
Immunoglobulin A	IgA
Inflammatory Bowel Disease	IBD

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Interleukin	IL
Interleukin-2	IL-2
Intestinal Epithelium Cells	IEC
Kilogram	Kg
Leukocyte Differential Count	LDC
Lipopolysaccharide	LPS
Low-density Lipoprotein	LDL
MacConkey Agar	MA
Microbial Associated Molecular Patterns	$MAMP_S$
Microfold Cells	M cells
Natural Killer Cell	NK Cell
New-Zealand White	NZW
Nucleotide Binding Oligomerization Domain Agents	NOD_S
Nutrient Agar	NA
Pattern Recognition Receptors	PRR
Periodic Acid Schiff	PAS
Potential of Hydrogen	PH
Proliferation-inducing Ligand	APRIL
Qualified Presumption of Safety	QPS
Rheumatoid Arthritis	RA
Scanning Electron Microscopy	SEM
secretory immunoglobulin A	S-lgA
SUNlike Protein	SLP
T helper cell	T_h
Thymus cell	T cell
Toll-Like Receptors	TLR_S
Tumour Necrosis Factor	TNF
Ultra Variable-Pressure Detector	UVD
White Blood Cell	WBC
World Health Organization	WHO

APPENDIX LIST

CHAPTER 1

INTRODUCTION

1.1 Introduction

Dietary factors believed to concede advantages to the immune system, and other adaptive functions. Generally, the term ‘immunonutrition’ is used to describe this interrelationship. Health benefits of certain nutrients in the diet have been reviewed long ago, including growth factors, secretory immunoglobulins, interferons, and lysozymes, which are known for their immunological advantages to the person. The immunomodulatory effects of minerals (especially, iron, selenium, and zinc), vitamin A (in conditions of compromised malnutrition), some amino acids, and nucleotides, in adaptive responses to the gut and in pathologic conditions (e.g. bowel disease, and necrotizing enterocolitis) have been discussed in several reports.

Health and immunity have been thought, since ancient times, to depend on nutrition. Certain types of food have recognized, aside from supplying sources of calories and nutrients, they uniquely provide prevention against nutritional diseases. Foods we consume are supplying nutritional sources for retaining good health and prevention of a wide range of diseases. Immunonutritions are known to effectively improve the outcome of patients, particularly those who are immunocompromised or malnourished, however, further research is still needed to assess the relationship between immune functions and immunonutrition in regard to those patients.

1.2 Background of the Study

Probiotics and certain herbal plant extractions, among others, have traditionally believed to enhance the immune system of those are consuming them. There is scientific evidence that probiotics and herbal plants can help to enhance the immune system, but further investigations are still required.

Many folklore medicines have claimed to naturally enhance the healing process of patients and boost their recovery. Numerous studies had referred to variety of plant extracts as they are associated with beneficial effectiveness on illness recovery. *Phaleria macrocarpa*, on of the local Malaysian plants, that is traditionally believed to contribute to the vitality of human health. It has been reported lately for numerous medicinal benefits, such as inflammatory and vasorelaxant activities (Altaf *et al.*, 2013), fungal illnesses (Garcia, 2015), bacterial infections (Gopalan *et al.*, 2015), anti-cancer (Soekmansto *et al.*, 2007), anti-oxidation (Lay *et al.*, 2014), wound

healing (Oliveira *et al.*, 2015). Moreover, recent studies on animal models have confirmed its anti-diabetic efficacy (Salih *et al.*, 2015).

Therefore, this study aims to synthesize and develop a natural probiotic using microorganisms isolated and identified from NZW rabbit colon, as well as, to prepare *P. macrocarpa* leaves extraction, and test these natural products on immunocompromised NZW rabbits to achieve; firstly, test the effectiveness of probiotic and *P. macrocarpa* extract on rabbits with weakened the immune system. Secondly, develop a nutritional supplement from available, local, cheaper, and safe resources, to replace the chemical medications (for their inevitable harmful side effects) with these supplements. Thirdly, assist the immunocompromised animals (or even human patients, in future) in maintaining their peripheral immune system. Fourthly, improve the immune cell response to be more effective than using conventional drugs or expensive commercial supplements.

1.3 Problem Statement

The use of probiotics is one of the current interests nowadays due to their effect on improving the general health and providing potential benefits to the host. There is increasing evidence that consume of probiotic products help to maintain immune responses, and thus, improve general immune defense mechanisms of host's body.

There are always groups of human individuals who admitted to hospital for major operation (e.g. tissue or organ transplants) and often given different types of

immunocompromising medications or immunosuppressant (such as, corticosteroids, calcineurin inhibitors, mTOR inhibitors, IMDH inhibitors) before and after the operation. In doing so, they are more susceptible to variety of infections and/or opportunistic pathogens. Moreover, there are also other group of individuals who are immunocompromised, such as pregnant females, those affected by AIDS or HIV, cancer patients who are subjected to chemotherapy, and some genetic disorders, they are mostly prone to severe complications and/or infections than others.

The current study validates the advantages of using a formulated probiotic and a local herbal plant extract (*P. macrocarpa* leaves). This was tested on immunocompromised NZW rabbits (as animal model of human disease), to investigate their capability in improving impaired immune system, in addition, to evaluate their role in maintaining colon histology which affected by the immunosuppressant.

1.4 Study Objectives

The objectives of this research are:

1.4.1 To evaluate the growth prefeormance, the microbial alterations of colon microflora, the immunological modifications of peripheral immune system, and the histopathological changes of colon tissue, due to the administration of Azathioprine in NZW rabbits.

1.4.2 To compare between the effectiveness of treatment with the novel synthetic probiotic and *P. macrocarpa* leaves extract on alterations of colon microbiota, immune system responsiveness, and colon histopathological changes in immunocompromised NZW rabbits.

1.4.3 To investigate any improvement after treatments with the probiotic and *P. macrocarpa* leaves extract on NZW immunocompromised rabbits.

1.5 Research Questions

The research questions of this study are:

1.5.1 What are the phytochemicals constituents, in general, present in *P. macrocarpa* leaves extract and their prospective activities?

1.5.2 How does the immunosuppressant drug, Azathioprine (Imuran®), affect growth performance of the control, immunocompromised without treatment, immunocompromised with the formulated probiotics treatment, and immunocompromised with *P. macrocarpa* extract treatment, in NZW rabbits groups?

1.5.3 How does the immunosuppressant drug, Azathioprine (Imuran®), affect the microbiota population within the gut of the control, immunocompromised without treatment, immunocompromised with the formulated probiotics

treatment, and immunocompromised with *P. macrocarpa* extract treatment, in NZW rabbits groups?

1.5.4 How does the immunosuppressant drug, Azathioprine (Imuran®), affect immune system response of the control, immunocompromised without treatment, immunocompromised with the formulated probiotics treatment, and immunocompromised with *P. macrocarpa* extract treatment, in NZW rabbits groups?

1.5.5 How does the immunosuppressant drug, Azathioprine (Imuran®), affect colon histopathological alterations of the control, immunocompromised without treatment, immunocompromised with the formulated probiotics treatment, and immunocompromised with *P. macrocarpa* extract treatment, in NZW rabbits groups?

1.6 Significance of the Study

Recently, studies have described the correlation between impaired immune functions and malnutrition. The researchers have established the association of nutritional deficiency, which always linked with impaired immune response, and cell-mediated immunity. So far, some malnutritional illnesses are predicted to reduce lymphocyte count and impair responses toward antigens. However, in this regards, certain probiotics and natural plant products are believed to maintain variety of peripheral immune mechanisms (Walsh *et al.*, 2011; Marano *et al*., 2013; Nieman *et al.*, 2013;

Cruzat *et al.*, 2014; Stefan *et al.*, 2014; Marco, 2015; Oliveira *et al.*, 2015; Mokoena *et al.*, 2016).

Moreover, immunocompromised individuals who are admitted to hospital are always tend to be more susceptible to infections (caused by microbial pathogens and/or opportunistic microorganisms) and resulting in serious illnesses (Vandenplas *et al.*, 2015).

Among all the corresponding literature review, no complete or well-designed study found with same objectives; which are testing natural probiotics and plant extracts in immunocompromised animals/humans to investigate their capability of improving immune system functions against infectious/opportunistic microorganisms, as well as, their effectiveness on colon histology, thus, this study has been suggested.

1.7 Limitations of the Study

This study conducted using NZW rabbits under standard laboratory controlled conditions (room temperature 25-30ºC, humidity 70-80%, automatic 12hr light-dark cycle, fed with standard pellets, and free access to water *libitum*). Therefore, it cannot generalize to all types of animals (or human).

1.8 Conclusion

Probiotics usually used to promote good health, since they inhibit the ability of harmful microbes to survive in the intestine, and so, prevent different diseases and illnesses. They could be obtained in a variety of dairy and non-dairy products and supplements. Current researches and studies are highly advised to focus on developing, synthesize, and testing new generations of effective probiotics. Meanwhile, *P. macrocarpa* extracts are frequently used in lots of traditional medical treatments, due to their highly contents of phenolic compounds. Several studies indicated that *P. macrocarpa* extracts have advantages to cure many medical illnesses and complications, however, none (up to date) has referred to its potential on maintaining gut microbiota, impaired immune system, and colon histology under immunocompromised conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Immunity refers to all physiological mechanisms (specific and non-specific) that provide the animal with the ability to recognize non-self materials as foreign, so to neutralize, eliminate or metabolize them with or without causing injury to its own tissues.

Immune system consists of certain organs, special tissues, specified cells, effective molecules, and genes (Shetty, 2005). If any biological system is invaded by foreign antigen, it will activate the host's immunity to ensure that all basic functions, of all systems in the body, work normally (Qing-guanga et al., 2011).