PILOT STUDY ON MICROBIOTA PROFILING IN FEMALE PATIENTS WITH AUTOIMMUNE CONNECTIVE TISSUE DISEASE FROM HOSPITAL PUTRAJAYA

by

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DISSERTATION IS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

2023

ACKNOWLEDGEMENT

First and foremost, I would want to thank God for providing me with confidence and support throughout all of the difficult moments of finishing my dissertation. I am very grateful for His unconditional and limitless love, compassion, and grace.

I would like to thank and profoundly appreciate my supervisor, Dr Nor Effa Syazuli Binti Zulkafli, for her continuous support, supervision, and advice during my dissertation. Her knowledge and patience have been invaluable to me and have played a critical part in the success of this project.

I am grateful to University of Science Malaysia for providing me with the opportunity to study higher education of master degree in Medical Research with a wide range of knowledge and skills for completing my research. Furthermore, I would like to extend a special thanks to Dr Asmida Binti Isa who played role as my academic advisor and provide numerous moral supports and sharing session which help me to gain more courage to finish this study.

I would also want to thank my friend Rammiya Rajasegaran for her love and encouragement while I was working on the dissertation. I would not have been able to accomplish this trip without her support, inspiration, and concern. I would like to acknowledge my family, especially my mother, Vijaya Lakshmy Ramasamy, and father, Wasdewa Kannan, for their steadfast support and understanding during my research and writing process. The prayers they offer for me have kept me going thus far. Finally, this study would not have been possible without the help of others who contributed directly or indirectly to its completion.

DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) for the Master of Science in Medical Research. It has not been sent to other universities. With that, this research can be used for consultation and photocopied as reference purposes.

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Matric No. : P-IPM0022/22

Date : 25 August 2023

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
µg/mL	Micrograms per millilitre
µL	Microlitre
g	Gram
mL	Millilitre
pmol/µL	Picomol per microlitre
rpm	Revolutions per minute
V/cm	Volt per centimetre

LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
ACPA	Anti-citrullinated protein antibody
AhR	Aryl hydrocarbon receptor
APCs	Antigen-presenting cells
AREE2	Antigen receptor response element 2
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool Nucleotide
cDNA	Complementary DNA
CIDP	Chronic inflammatory demyelinating polyneuropathy
CRF	Clinic Record Form
CTD	Connective tissue disease
dNTP	Deoxynucleoside triphosphate
ds-DNA	Double stranded Deoxyribonucleic acid
DC	Dendritic cell
DM	Dermatomyositis
DNA	Deoxyribonucleic acid
EB	Epidermolysis bullosa
EBV	Epstein-Barr virus
EDS	Ehlers-Danlos syndrome
EtBr	Ethidium bromide
FRGS	Fundamental Research Grant Scheme
Foxp3	Forkhead box P3
GBS	Guillain-Barré syndrome
GITR	Glucocorticoid-induced TNFR-related protein

HLA	Human leukocyte antigens
HP	Hospital Putrajaya
ICF	Informed Consents Form
IFN	Interferon
IgA	Immunoglobulin A
IIUM	International Islamic University of Malaysia
IL-10	Interleukin-10
IL-22	Interleukin-22
IREC	IIUM Research Ethics Committee
LD	Linkage disequilibrium
LYP	Lymphoid phosphatase
mRNA	Messenger RNA
MCTD	Mixed connective tissue disease
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MREC	Medical Research Ethics Committee
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T cells
NGS	Next generation sequencing
NK	Natural killer
NMRR	National Medical Research Register
NOD1	Nucleotide-binding oligomerization domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	PEST-domain Enriched Phosphatase
PSA	Polysaccharide A
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22

PWV	Pulse wave velocity
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RORt	Receptor-related orphan receptor gamma t
ROS	Reactive oxygen species
RT-dPCR	Real Time Digital Polymerase Chain Reaction
SCFAs	Short-chain fatty acids
SFB	Segmented filamentous bacteria
SLE	Systemic lupus erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SSc	Systemic sclerosis
TAE	Tris-acetate EDTA
TCR	T cell receptor
Th1	T helper 1
TCDD	2,3,7,8-tetrachloro dibenzo-p-dioxin
UCLA	University of California, Los Angeles
USA	United State of America
USM	University of Science Malaysia

KAJIAN PERINTIS MENGENAI PROFIL MIKROBIOTA DALAM KALANGAN PESAKIT WANITA YANG MENGHIDAP PENYAKIT TISU PENGHUBUNG AUTOIMUN DARI HOSPITAL PUTRAJAYA

ABSTRAK

Penyakit autoimun berlaku di dalam tubuh manusia apabila sistem pertahanan tubuh tidak dapat membezakan atau mengenali antara antigen asing dan sel sendiri dan dengan itu, menyerang dan memusnahkan sel dan tisu yang sihat di dalam badan sendiri. Penyakit tisu penghubung autoimun (CTD) adalah penyakit yang sistem pertahanan badan mempengaruhi fungsi dan struktur tisu penghubung, seperti sendi, kulit, mata, trek gastrointestinal, jantung, dan paru-paru kebanyakannya di badan wanita. Usus mikrobiota berperanan dalam sistem pertahanan manusia dengan memberikan perlindungan terhadap patogen. Walau bagaimanapun, ketidakseimbangan mikrobiota usus berlaku pada pesakit autoimun menimbulkan soalan mengenai sama ada mikrobioma dalam usus menyebabkan autoimun di dalam badan atau sebaliknya. Oleh itu, kajian ini bertujuan untuk menunjukkan kehadiran mikrobiota usus dalam pesakit wanita yang mempunyai tisu penghubung autoimun (CTD) berbanding dengan wanita yang sihat. Sampel najis dan sampel darah dikumpulkan dari wanita yang sihat dan pesakit autoimun dan DNA diekstrak dari sampel najis menggunakan QIAamp PowerFecal Pro DNA. Selanjutnya, DNA yang diekstrak diuraikan menggunakan 16S rRNA next generation sequencing (NGS). Urutan yang dihasilkan diselaraskan dan dianalisis menggunakan Basic Local Alignment Search Tool (BLAST) dan mikroba yang dikenal pasti diprofilkan menggunakan pelayan web MicrobiomeAnalyst dan menhasilkan 'heatmaps' bagi subjek sihat dan pesakit autoimun. Perbandingan 'heatmaps' antara subjek sihat dan

pesakit menunjukkan kedua-dua kategori mempunyai kelimpahan Blautia argi (prevalensi 1.0) yang menunjukkan pesakit tidak mempunyai ketidakseimbangan Blautia argi dan juga ia tidak menyebabkan autoimun di badan pesakit. Selanjutnya, jika dibandingkan dengan subjek sihat, pesakit mempunyai banyak Bifidobacterium, dan Lachnospiraceae genera (prevalensi 1.0) yang tidak normal dan boleh menyumbang kepada autoimun. Kelimpahan tinggi dalam Streptococcus genera juga terdapat pada sampel pesakit yang mempunyai kemampuan peniruan molekul dalam menghasilkan 11 protein homolog yang serupa dengan protein manusia yang dapat menyumbang kepada pengembangan autoimun dalam badan seseorang. Kajian semasa menunjukkan hanya 'heatmaps' yang menunjukkan ketidakseimbangan mikrobiom usus dalam sampel pesakit, tetapi, ia tidak memberikan pemahaman atau bukti sebab ketidakseimbangan, autoimun atau pengaruh mikrob usus dalam sistem pertahanan badan. Oleh itu, kajian lanjut adalah perlu untuk menganalisis peranan mikrobiom usus dalam sistem pertahanan badan, bagaimana mikrob mempengaruhi aktiviti sistem pertahanan badan, bagaimana ia menyebabkan autoimun dalam seseorang dan menentukan kesan sebenar dysbiosis terhadap perkembangan penyakit ini.

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ABSTRACT

An autoimmune disease occur in human body when the immune system could not differentiate or recognise between foreign antigen and own cells and thus, attacks and destroys healthy cells and tissues in the own body. Autoimmune connective tissue disease (CTD) is a disease when the immune system affect the function and structure of connective tissues, such as joints, skin, eyes, gastrointestinal track, heart, and lungs mostly in female body. Gut microbiota play ab important role in human immune system by providing protection against pathibionts. However, imbalance of gut microbiota occur in autoimmune patients resulting in curiosity whether the microbiome in the gut cause autoimmunity in the body or vice versa. This study therefore aimed to profile presence of gut microbiota in healthy versus autoimmune connective tissue disease (CTD) female patients. The stool samples and blood samples were collected from healthy and autoimmune patients and the DNA were extracted from stool samples using QIAamp PowerFecal Pro DNA kits. Furthermore, the extracted DNA was sequenced using 16S rRNA next generation sequencing (NGS). The produced sequences was aligned and analysed using Basic Local Alignment Search Tool (BLAST) and the identified microbes was profiled using MicrobiomeAnalyst webserver and produced heatmaps of healthy control and autoimmune patients. The comparison of heatmaps between the healthy control and patients showed both category have high abundance of *Blautia argi* (prevalence 1.0) which indicate the patients does not have imbalance of *Blautia argi* and also it does

not cause the autoimmunity in the patients body. Furthermore, compared to healthy control, patients have high abundance of *Bifidobacterium*, and unclassified *Lachnospiraceae* genera (prevalence 1.0) which is abnormal and may contribute to autoimmunity. High abundance in Streptococcus genera also found in patients samples that have molecular mimicry ability in producing 11 homologous proteins similar to human proteins which may contribute to development of autoimmunity. The current study showed only the heatmaps that indicate the imbalance of gut microbiome in the patients samples, but, it does not provide the understanding or evidence of causes of imbalance, causes of autoimmunity or influence of gut microbes in immune system. Thus, gut microbiota in CTD patients from Hospital Putrajaya and healthy control have been profiled and it serve as fundamental to establish the correlation between autoimmune and gut dysbiosis among Malaysian population.

CHAPTER 1

INTRODUCTION

1.1 Research background

Immune system in human play important role in protecting body from infection and diseases cause by microbes or foreign antigens that diffuse into the body, such as bacteria, and viruses. The immune system can recognise and differentiate the foreign materials from the body cells, and eliminate the materials under certain mechanisms. Most of the cells that play role in immune system are produced from the bone marrow, such as B cells and thymus, such as T cells. The cells in the body consists of varies receptors which play role in detecting pathogens or any infection evidence (Nicholson, 2016). However, certain pathogens, such as viruses have properties to alter the structure of receptor on the membrane cells which cause difficulties in recognise as foreign antigen. In this case, the receptors in the cytoplasm play role by binding to the ligand of virus and trigger a signal that indicate presence of pathogen. Human cell have advance system that use for screening and production of protein to differentiate the protein are not produced by foreign antigens like viruses. However, if a protein from foreign antigen is recognised, it trigger adaptive immunity mechanisms by producing cytokines which serve as alarm signals and stimulate apoptosis of the cells (Nicholson, 2016).

Tackling infections also one of the action of immune system in the body. Almost half of the cells with tackling property are involve in innate immune system, such as neutrophils and macrophage. These cells produce effective substances, such as enzymes which is very destructive by digest proteins and reactive oxygen species (ROS). The phagocytosis processes occur by engulf and digest the protein or foreign

materials that they damaged. However, sometimes some microbes can escape or not effectively destroy by cells from innate immune system. Thus, these trigger the mechanism of action of lymphocytes from adaptive immune system. After the foreign antigen destroyed, B cell and T cell store memory of infection for fast and effective counterattack towards the same infection in the future (Nicholson, 2016).

An autoimmune disease occur in human body when the immune system in body could not differentiate or recognise between foreign antigen and own cells and thus, attacks and destroys healthy cells and tissues in the own body. In autoimmune disease, the immune system recognise proteins on tissue cells like joints or skin as foreign materials and release protein called autoantibodies to attack the healthy cells of tissues. Some autoimmune diseases only affect one part of body or organ, while some can effect multiple part of the body or organs (Watson, 2019). There are several autoimmune diseases based on target location in the body, such as autoimmune connective tissue disease (CTD), digestive autoimmune diseases, autoimmune endocrine disease, autoimmune skin disease, autoimmune neurology and other diseases (Watson, 2019).

In autoimmune connective tissue disease, the most common and specific diseases, such as psoriatic arthritis, rheumatoid arthritis (RA), Sjögren's syndrome, and systemic lupus erythematosus (SLE). In digestive autoimmune diseases, Crohn's disease, celiac disease, and ulcerative colitis are the most common diseases. Furthermore, autoimmune endocrine disease consists of Graves' disease, Hashimoto's thyroiditis, and Addison's disease, while in autoimmune skin disease, dermatomyositis and psoriasis are the most autoimmune diseases (Catinean *et al.*, 2019). Apart from that, chronic inflammatory demyelinating polyneuropathy (CIDP),

Guillain-Barré syndrome (GBS), and multiple sclerosis (MS) are most common autoimmune diseases in nervous system. Other common autoimmune diseases are such as Type 1 diabetes, vasculitis, myasthenia gravis, and pernicious anemia (Talotta *et al.*, 2017).

Connective tissues in the human body are made of two major proteins, such as collagen and elastin. Collagen can be found in the ligaments, tendons, skin, cartilage, cornea, bone, and blood vessels. While elastin can be found in ligaments and skin which characterized as stretchy protein like rubber band. Connective tissue is a biological tissue contain extracellular matrix that support, bind, and protect organs in the body (Fawcett, 2019). Connective tissue disease (CTD) is a disease that affects the function and structure of connective tissues, such as joints, skin, eyes, gastrointestinal track, heart, and lungs. In CTD disease, the collagen and elastin are affected and injected by inflammation (Rao and Bowman, 2013). The CTD disease categorized by two type, such as genetic (inheritance) caused by mutation and autoimmune disease causes inflammation of connective tissues. Example of CTD diseases caused by genetic are Ehlers-Danlos syndrome (EDS), epidermolysis bullosa (EB), Marfan syndrome, and osteogenesis imperfecta while autoimmune CTD disease are polymyositis, dermatomyositis, rheumatoid arthritis (RA), scleroderma, Sjogren's syndrome, systemic lupus erythematosus (SLE), and vasculitis. This study focuses on types of CTD disease which is autoimmune connective tissue disease (Young, 2018).

Apart from this, there is another type of disease which is mixed connective tissue disease (MCTD). Mixed connective tissue disease (MCTD) is a very rare systemic inflammatory rheumatic condition. It also known as overlap syndrome

which indicates a patient has more than one inflammatory rheumatic disease. It is also categorized as autoimmune CTD disease (Benjamin *et al.*, 2018). There are several symptoms of autoimmune CTD diseases can be seen in the body, such as joint pain, muscle weakness, numb fingertips, and swelling in the fingers, difficulty swallowing, fatigue, weight loss and fever. Other than that, cough, red spots on the skin, ulcers, nodules, thick skin and organ damage are symptoms of autoimmune CTD diseases can be seen in the body (Dunkin, 2021).

According to Malaysian SLE Association (2020), women are at high risk of autoimmune connective tissue disease like SLE which is 90% of SLE patient are women while only 10% of men and children have risk getting SLE. Normally, women in childbearing age have high risk which age range between 15 to 50 years old. In certain Western countries, 1 in 250 to 500 people have risk of autoimmune CTD diseases especially among Afro-Caribbean. In United State of America (USA), 1 in 2000 people has risk of autoimmune CTD diseases, whereas, in China, 1 in 1000 people has risk of autoimmune CTD diseases among people. In Malaysia, compared to other autoimmune CTD diseases, SLE are recorded as high risk disease which is more than 10,000 people were diagnosed over past 30 years (Malaysian SLE Association, 2020).

Even though the cause of autoimmune connective tissue disease is still unknown, there are certain factors that contribute to autoimmunity, such as genetic, defective regulation and environment. According to Rosenblum *et al. (2015),* in genetic factor, polymorphisms is located at varies regulatory regions of genes which encode or produce functional protein or substances for immune responses. Human leukocyte antigen (HLA) class II alleles are found to be contributed to autoimmune

disease. However, it is questionable that how different HLA alleles cause any other autoimmune disease and exhibiting the autoantigens which attract by self-reactive T cells because HLA alleles have ability in displaying self-antigens in common human body. Thus, most of autoreactive T cells in human body have ability to escape from thymic deletion. Other than that, the relationship of cytokines and cytokines receptor genetic polymorphisms has connection to many autoimmune diseases (Rosenblum *et al.*, 2015). For example, presence of genetic polymorphisms in IL-23R gene which increase proinflammatory capacity of Th17 cells are discovered in scleroderma, ankylosing spondylitis, Behcet's disease, and psoriasis arthritis (Gourh *et al.*, 2020). These lead to tissue damage and targeting monoclonal antibodies specific for either p40 or IL-17A (Papp *et al.*, 2012).

In defective regulation, failure in self-tolerance in abnormality immune cells occur mostly in autoimmune diseases include CTD. However, this rise question that which and the reason of self-tolerance mechanisms fail in specific diseases. Patients with SLE have defection in immaturation of B cells in the bone marrow, defection of receptor editing, and control in differentiate of B cells maturation in peripheral tissues. Production of autoantibodies by mature B cells occurs in people with SLE before detection or presence of antigen which indicate that defection in tolerance of B cell cause development of autoimmunity. Imbalance between effector T cells and functional Treg cells occur in T cell–dependent inflammatory autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriatic arthritis (Rosenblum *et al.*, 2015). In addition, failure of regulation cause autoimmunity in human which have been analysed experimentally that abnormalities in the self-antigens are associated with autoimmune reactions. Antigen-presenting cells (APCs) produce proteins or denatured protein extracellularly which lead to rise

of peptide/MHC complexes that different from production of protein inside of APCs. These lead to activation of pathogenic T cells. This activation of self-reactive T cells occur due to recognition of conformational isomers of peptide/MHC or from a differential binding register of a peptide within the channel of major histocompatibility complex (MHC) molecule. However, it is still unknown that the alteration of self-antigen causes autoimmunity in human body (Mohan *et al.*, 2011).

Infection is play environmental role that trigger autoimmune connective tissue disease. Multiple studies shows that the infection does trigger autoimmunity with explanation of epitope spreading, antigenic complementarity, and excessive innate/pattern recognition receptor activation (Root-Bernstein and Fairweather, 2014). Example of study showed that Epstein-Barr virus (EBV) infection has been associated with multiple sclerosis (MS) through postmortem of brain tissue (Rosenblum *et al.*, 2015). By increasing response of myelin-specific T cell, systemic infections trigger relapses with relapsing-remitting MS in patients with MS, periodontal infections and rheumatoid arthritis (Mikuls *et al.*, 2014). Some infection play role in protecting body from autoimmune disease. For example, infection of *Bacteroides fragilis* have been discovered that it play role as protector against experimental autoimmune encephalomyelitis by induction of Treg cells. However, as the infection of *Bacteroides fragilis* decreased the incidence of MS increase (Belkaid and Hand, 2014).

1.2 Problem Statement

Although there are three possible factor that may cause autoimmune connective tissue disease, it is still not confirmed that the main source of cause in environmental factor which is infection by microbiome in the gut. Gut microbes has

important roles in immune system by protecting human body against pathobiont. However, in the case of autoimmune connective tissue disease, the microbiome in the human gut becomes imbalance. Imbalance of microbiome in the gut occur by decreased in large quantity of beneficial commensal genera, such as *Faecalibacterium*, *Clostridium* and *Bacteroides* and increased in large quantity of pathobiont genera, such as *Fusobacterium*, *Prevotella* and *Erwinia*. This arise a curiosity whether the microbiome in the gut cause autoimmunity in the patients with CTD disease or the autoimmunity trigger by other factor cause imbalance of gut microbiota which later affect the tissue in the body.

By using 16S rRNA sequencing technique, we attempted to identify and profile the gut microbiota between female patients with healthy body and autoimmune connective tissue disease. This is hope to serve as fundamental to establish the correlation between immune system and gut dysbiosis among Malaysian population.

1.3 Objective

The objective of this study was to profile presence of gut microbiota in healthy versus autoimmune connective tissue disease patients by 16S rRNA next generation sequencing (NGS).

CHAPTER 2

LITERATURE REVIEW

2.1 Function of gut microbiota in human immune system.

The microbiota in the gut has symbiotic relationship with human immune system. These is acceptable because the gut microbiota give beneficial reaction by providing protection against pathobiont, maintain mucosal microenvironments of gut, and metabolized digested nutrients, drugs and substances. For the usage of metabolisms, gut microbiota help to ferment and digest carbohydrate into short-chain fatty acids (SCFAs) for easy to absorb, and synthesize vitamin which help to regulate the gut-associated immune cells. Development of immune system required gut microbiota to provide essential function in the immune system and metabolisms and in return, the microbiota in the gut protected from human immune defense. These indicate the presence of gut microbiota is important to regulate function of systemic immune system in the body (Figure 2.1) (Zulkafli *et al.*, 2019).

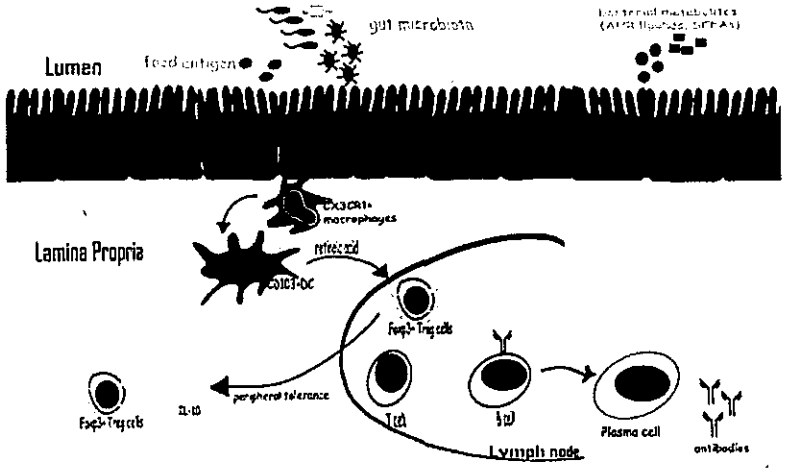

Figure 2.1. Modulation of intestinal immune homeostasis by gut microbiota (Zulkafli *et al.*, 2019).

2.1.1 Microbiota and innate immune homeostasis

The capacity of intestinal APCs, which evolved alongside the microbiota, to defend the host against infection while preserving immunological tolerance to normal gut flora is critical. Dendritic cells (DCs) of Peyer's patches for example, produce much more interleukin-10 (IL-10) than splenic DCs stimulated under equivalent conditions. When exposed to microbial stimuli in homeostatic situations, gut macrophages, like DCs, exhibit a unique phenotype defined as "inflammation anergy," which refers to intestinal macrophages' noninflammatory character (Wu and Wu, 2012).

Immune tolerance is a condition of unresponsiveness in which the lymphocytes remain alive but cannot perform effector activities against a specific antigen. There are two type of immune tolerance, which are central tolerance and peripheral tolerance. Lymphocytes containing self-antigen receptors are eliminated at an early point in lymphoid cell development. This mechanism known as the central tolerance gives permit to self-reactive B and T cells to be eliminated. Apoptosis occurs in lymphocytes that do not receive survival signals. When a subset of T cell receptors receives the right antigen signals, they exit the thymus and circulate in the periphery. Some CD4+ T cells are selected in the thymus to develop into "natural" T regulatory cells (nTregs), which express the FoxP3 transcription factor and dampen the immune response through both direct and indirect mechanisms (Benlaribi *et al.*, 2022).

Peripheral tolerance ensures that self-reactive T cells are eliminated or become anergic when they escape into the periphery. Three mechanisms can lead to peripheral tolerance. The first process is anergy induction, which refers to a state of inactivation

in which lymphocytes are alive but unable to react to antigen. For example, if dendritic cells are not properly activated and transmit self-antigen to T cells without costimulation and cytokine signals, T cells that are tolerant or anergic to that self-antigen are produced. The second mechanism is the apoptosis-mediated elimination of autoreactive T cells. The last mechanism is the production of "induced" regulatory T cells (Tregs) in the peripheral tissues. T cells exposed to the cytokine TGF-beta can differentiate into "induced" T regulatory cells (Tregs). The Tregs that have been "induced" have comparable effector roles to natural Tregs, however they are created in the peripheral rather than the thymus. (Scheinecker *et al.*, 2019).

Numerous studies provide substantial evidence of the gut microbiota's critical role in limiting the formation of APCs. There were fewer intestinal but not systemic DCs in GF animals, and monocolonization of GF mice with *Escherichia coli* was enough to recruit DCs to the intestines. Furthermore, it has recently been revealed that ATP generated by microorganisms can activate a subset of DCs with CD70 and CX3CR1 on their surface, triggering the formation of Th17 cells. Intestinal macrophages make up the majority of the body's tissue macrophage population. Furthermore, GF mice's peritoneal macrophages have been shown to have reduced chemotaxis, phagocytosis, and microbicidal activity (Wu and Wu, 2012).

The innate immune system is strongly reliant on neutrophils, and studies have revealed that the microbiome has a large influence on how neutrophils are controlled. Neutropenia is one of the most noticeable characteristics of GF rats. Furthermore, GF rats' peripheral blood neutrophils had lower phagocytic activity as well as lower superoxide anion and nitric oxide generation. A recent mechanistic investigation discovered that cytosolic receptor-nucleotide oligomerization domain 1 (NOD1)

recognition of peptidoglycan from the gut microbiome increased bone marrow neutrophil killing activity. This data expertly showed how gut microbiota might influence systemic immunity (Wu and Wu, 2012).

Traditional natural killer (NK) cells are innate lymphocytes capable of recognising and killing infected and altered target cells by secreting interferon (IFN) or perforin. Two NK cell subtypes have recently been found in the gastrointestinal mucosa that express the NK cell natural cytotoxicity receptor NKp46. One kind of gut NKp46+ cell closely resembles typical NK cells, whereas the other produces less IFN and lacks perforin. These atypical gut NKp46+ cells are distinguished from standard NK cells by the expression of the nuclear hormone receptors retinoic acid receptor-related orphan receptor gamma t (RORγt) and interleukin-22 (IL-22). Because GF mice lack NKp46+ cells that generate IL-22, this suggests that the gut microbiota is important in promoting IL-22+NKp46+ cells (Wu and Wu, 2012).

2.1.2 Microbiota and adaptive immune homeostasis

$CD4^+$ T cells are an important component of the adaptive immune system. The bulk of the intestinal $CD4^+$ T cells are found in the LP. After stimulation, naive $CD4^+$ T cells can differentiate into one of four distinct subtypes: T helper 1 (Th1), Th2, Th17, or regulatory T cell (Treg). These many $CD4^+$ T cell subtypes may be distinguished by the transcription factors and cytokines they express (Figure 2.2). The regulation and balance of T-cell subtypes has a significant impact on one's health. Unregulated Th responses can be harmful, since the Th1 and Th17 responses have been related to autoimmune disorders and the Th2 response to allergic reactions (Wu and Wu, 2012).

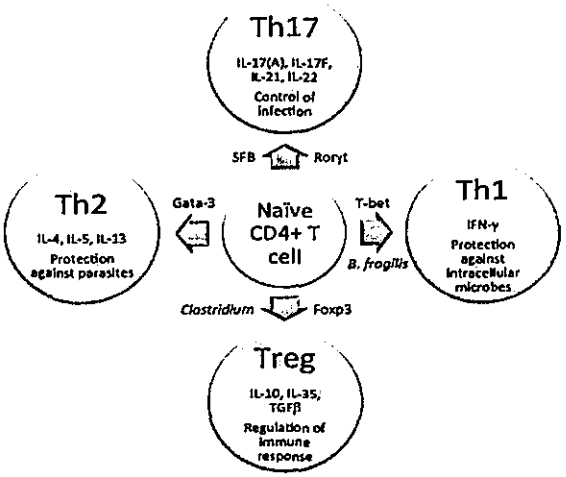

Figure 2.2. $CD4^+$ T cell differentiation is induced by commensal microorganisms. The four main cell types that can develop from naive $CD4^+$ T cells are Th1, Th2, Tregs, and Th17 (Wu and Wu, 2012).

The gut microbiota is essential for the production of $CD4^+$ T cells both inside and outside the intestine. As a result, GF mice have much less LP $CD4^+$ cells. GF animals have malformed spleens and mesenteric lymph nodes because they lack lymphocyte zones. Furthermore, GF mice exhibit a Th1/Th2 imbalance, indicating that their immune system is prone to the Th2 response. Recent studies even discovered a relationship between the development of several T-cell subtypes and a specific bacterial species. The polysaccharide A (PSA) molecules generated by *Bacteroides fragilis* have been shown to induce a systemic Th1 response. In contrast, segmented filamentous bacteria (SFB) were revealed to be potent LP Th17 cell inducers (Wu and Wu, 2012).

Clostridia, specifically members from cluster IV and XIVa, have been demonstrated to be capable of stimulating the induction of colonic Tregs. In another work, TLR9 signaling triggered by DNA from the gut microbiota was demonstrated to

preserve immunological homeostasis by preventing Treg cell conversion in the intestinal locations. Interestingly, *B. fragilis* PSA can signal via TLR2 on Tregs, suppressing a Th17 response. Furthermore, colonic Tregs had low amounts of the transcription factor Helios, which is a possible hallmark for thymus-derived Tregs. It is possible for T cells with a TCR specific for the colon to cause colitis if they fail to mature into Tregs and instead become T effector cells. Together, their data imply that the majority of colonic Tregs are of peripheral ancestry and are trained to be tolerant to commensal-derived foreign antigens by the gut microbiota (Wu and Wu, 2012).

2.2 Relationship between gut microbiota and autoimmune diseases.

According to Xu *et al.* (2019), aryl hydrocarbon receptor (AhR) might be the cause of autoimmunity in certain diseases which play role in signaling pathway either extrinsic or intrinsic into cellular responses. In an experiment using mice, low level of innate IL-22 levels in AhR-deficient mice cause growth of commensal segmented filamentous bacteria (SFB) that lead to proliferation of T helper 17 (Th17) cell. AhR play role in protecting T-cell-mediated by suppress pathogenic activity of Th17 cells (Qiu *et al.*, 2013). In addition, 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD) known as AhR ligand cause growth of SFB and *Bacteroides fragilis* in mice which analysed by comparing the level of traditional gut microbiome. This analysis significantly helped that the TCDD activity can be regulated by gut microbiome which is SFB that demonstrating therapeutic potential between AhR ligands (Stedtfeld *et al.*, 2017).

Although the autoimmune caused by gut microbiota remain unclear, but imbalance microbial community in gut has been identified causing autoimmune diseases. The dysbiosis cause post-translational in alteration of autoantigens and cross reaction with autoantigens at molecular level. The gut bacteria can move through

defective gut barrier which causes interaction with immune cells and tissue cells and lead to systemic autoimmunity (Dehner *et al.*, 2019).

The asymmetry relationship between the gut microbiota and autoimmune disease due to several mechanisms gave negative effect to human immune system and function. Induction of antigen presentation and production of cytokine due to activation of antigen-presenting cells (APCs) can affect the function of T cell. This gives negative impacts by disrupting the homeostasis between Th17 cells and Treg cells. The similarity between foreign antigens and self-antigens have mimicry properties that causes T and B cells activation and inducing autoimmunity (Xu *et al.*, 2019). The imbalance microbiome in the gut may develop diseases in patients with autoimmune diseases (Figure 2.3). These causes molecular mimicry, reduction of intestinal mucosa permeability, and the host immune response caused by the microbiota, and antigenic mimicry (Xu *et al.*, 2019).

Autoimmune diseases and alteration of the gut microbiota composition.

Diseases	Species	Methods	Increasing microbiota species	Decreasing microbiota species
RA	Mouse	16S rRNA gene sequencing	*Dasulfovibrio* *Mucispirillum* *Helicobacter* Lachnospiraceae *Rikenellaceae_RC9*	*S24-7* *Rikenella*
	Human	16S rRNA sequencing		Euryarchaeota
	Human	16S ribosomal DNA	*Collinsella*	Actinobacteria
	Human	Denaturing gradient gel electrophoresis	*Lactobacillus*	
	Mouse	16S rRNA sequencing	Bacteroidaceae *Lachnospiraceae* S24-7	

Figure 2.3. Modification of microbiome in gut with correspond autoimmune disease. (Xu *et al*, 2019, pp. 4 – 5).

2.2.1 Composition of gut microbiota associated in lupus.

Mutation of Fas^{lpr} in mice causes systemic autoimmunity, swelling of lymph nodes, and kidney inflammation (Andrews, 1978). The mice with systemic autoimmunity death in one week caused by increased level of autoantibodies and immune complexes. The analysis showed that the death mice had reduction in level of family *Lactobacillaceae* and increase level of family *Lachnospiraceae* by more than 65% of microbiome in the gut. The *Lachnospiraceae* family contains anaerobes known as *Clostridium* cluster XIVa which produce butyrate such as *Butyrivibrio* and *Roseburia*. Overall, the level of *Clostridiales* family XIII, and the *Streptococcaceae* were high in lupus-prone mice. The analysis showed that the lupus-prone mice have varies bacterial content and diversity compared to healthy controls, which indicate that lupus may manipulate gut microbiota (Zhang *et al.*, 2014).

Another study found that the species *Streptococcus*, *Campylobacter*, and *Veillonella* are positively associated with SLE disease activity and negatively associated with the genus *Bifidobacterium*. *Clostridium sp.* ATCC BAA-442, *Atopobium rimae*, *Shuttleworthia satelles*, *Actinomyces massiliensis*, *Bacteroides fragilis*, and *Clostridium leptum* were discovered to be enriched in non-treatment SLE patients and largely restored following therapy. In line with the previous study, our findings demonstrated an increase in *Ruminococcus gnavus* in the guts of lupus nephritis patients. These surprising findings suggest that the gut environment is out of balance in SLE disease, emphasising the importance of researching associated immune-microbiota interactions (Figure 2.4) (Li *et al.*, 2021).

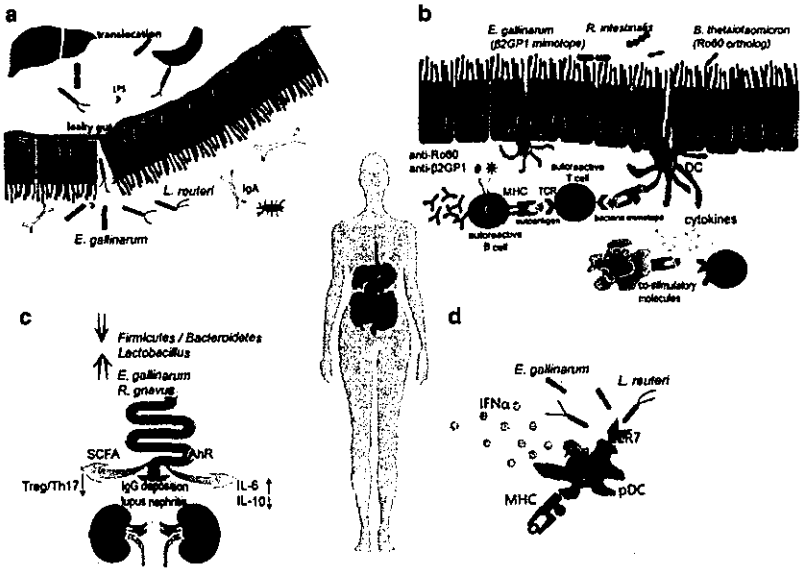

Figure 2.4. Variable microbiota in the gut cause lupus which called butterfly effect (Li *et al.*, 2021).

2.2.2 Alteration of microbiota in gastrointestinal tract for systemic sclerosis (SSc).

According to Volkmann (2017), the experimental observation of gastrointestinal tract microbiota was done to the patients with systemic sclerosis (SSc) who undergoes colonoscopy compared with healthy people which recorded that commensal genera such as *Faecalibacterium*, *Clostridium* and *Rikenella* which have beneficial role in body were decreased while pathobiont genera, such as *Fusobacterium*, *Prevotella*, *Ruminococcus*, *Akkermansia* and the uncommon γ-*Proteobacteria*, *Erwinia* and *Trabsulsiella* were increased in the patient with SSc (Figure 2.5) (Volkmann, 2017).

Other than that, patients with SSc also found contain low concentration of commensal genera, *Bacteroides* and *Faecalibacterium*, and high concentration of

pathobiont genera, such as *Fusobacterium*, *Ruminococcus* and *Akkermansia*. The two observation concluded that *Ruminococcus* and *Akkermansia* genera are recorded as highest pathobiont genera found in the SSc patients. In addition, *Ruminococcus* and *Akkermansia* genera produce fibrotic phenotype in patients with Crohn's disease. Thus, this result showed that these two genera and its production of metabolisms are responsible in producing fibrosis feature in SSc patients (Volkmann, 2017).

Table 1. Increased and decreased microbial taxa in systemic sclerosis patients versus controls

Study	Region	Design/sample	N	Increased in SSc[a]	Decreased in SSc[a]
Volkmann *et al.* [25•]	Los Angeles, USA	Cross-sectional/colonic lavage sample	17[b]	*Lactobacillus; Bifidobacterium; Fusobacterium; Erwinia; Ruminococcus Prevotella*	*Faecalibacterium; Clostridium; Rikenella*
Volkmann *et al.* [26•]	Oslo, Norway	Cross-sectional/fecal sample	17	*Lactobacillus*	*Clostridium; Bacteroides*
Volkmann *et al.* [26•]	Los Angeles, USA	Cross-sectional/fecal sample	17[b]	*Lactobacillus; Fusobacterium; Erwinia; Ruminococcus*	*Faecalibacterium; Bacteroides*
Andréasson *et al.* [28••]	Lund, Sweden	Cross-sectional/fecal sample	98	*Lactobacillus*	*Faecalibacterium prausnitzii; Clostridiaceae*
Bosello *et al.* [29]	Rome, Italy	Cross-sectional/fecal sample	66	*Lactobacillus; Ruminococcus; Roseburia; Faecalibacterium*	*Clostridium; Odoribacter; Veillonella; Prevotella*

Figure 2.5. Alteration of gastrointestinal microbiota in systemic sclerosis (SSc) patients (Volkmann, 2017, p. 3).

However, there are large quantity of commensal genera, such as *Faecalibacterium* and *Bacteroides* in the SSc patients from Norwegian compared with SSc patients from University of California, Los Angeles (UCLA). These indicate the alteration of microbiota in the gastrointestinal tract of SSc patients are different based on the region or location of the patients live. The different alteration of gastrointestinal microbiota in SSc patients may due to genetic differences, dietary variation, differences in SSc severity, and phenotypic expression of SSc (Figure 2.6) (Volkmann, 2017).

Table 2. Summary of microbial taxa associated with systemic sclerosis-gastrointestinal tract symptoms

Study	Region	Design/sample	N	Increased GIT-symptoms[a]	Decreased GIT-symptoms[a]
Volkmann *et al.* [25•]	Los Angeles, USA	Cross-sectional/ colonic lavage sample	17[b]	*Fusobacterium* *Actinobacillus*	*Bacteroides fragilis* *Candidatus arthromitus* *Clostridium*
Volkmann *et al.* [26•]	Oslo, Norway & Los Angeles, USA	Cross-sectional/ fecal sample	17[b]	*Prevotella; Porabacteroides*	*Clostridium* *Lactobacillus*
Volkmann *et al.* [38]	Los Angeles, USA	Longitudinal/ fecal sample	17[b]		*Bacteroides*

Figure 2.6. Alteration of gastrointestinal tract microbiota in SSc patients based on region (Volkmann, 2017, p. 5).

2.2.3 Relationship between gut microbiota and rheumatoid arthritis (RA).

According to Maeda and Takeda (2017), there are more than 100 genetic loci are responsible in causing rheumatoid arthritis (RA). Thus, it is difficult to understand the cause of RA in the human body. An immunoglobulin A (IgA) anti-citrullinated protein antibody (ACPA) was detected before the symptoms of arthritis began. This finding showed that the RA is originally begun at mucosal sites, such as oral cavity and the gut. As we know, the major pathogenic cause of RA is *Porphyromonas gingivalis* because this is the only bacteria that produce peptidylarginine deiminase which may trigger ACPA (Figure 2.7) (Maeda and Takeda, 2017).

By using flow cytometry, 16S ribosomal ribonucleic acid (rRNA) hybridization, and deoxyribonucleic acid (DNA) staining, the microbiota that contained in the gut was analysed in the RA patients showed decreased of the *Bacteroides fragilis* subgroup, the genus *Bifidobacterium*, and *Eubacterium rectale–Clostridium coccoides.* Furthermore, scientists also found *Prevotella copri* in human

faecal sample of RA patients from Japan in the absence of human leukocyte antigen (HLA)-DRB1 (Maeda and Takeda, 2017).

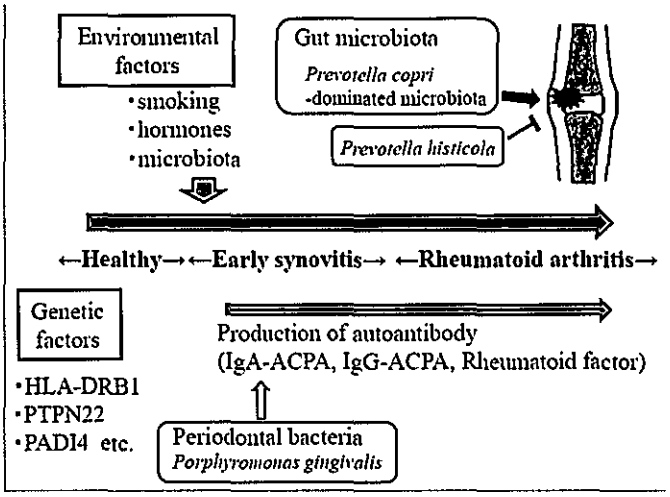

Figure 2.7. Development of RA caused by gut microbiota (Maeda and Takeda, 2017).

2.2.4 Alteration of gut microbiome in patients with dermatomyositis (DM)

The aetiology of dermatomyositis (DM) is tightly linked to damage to the microvasculature, and mounting data suggests that the gut microbiota may have an impact on vascular disease. In fact, microbiome-related variables contributed more to the variation in carotid-femoral pulse wave velocity (PWV) than did well-known metabolic syndrome indicators. *Ruminococcaceae* family loss and decreased microbiome diversity were noted. Patients' enhanced production of molecule adhesion and higher blood levels of endothelial injury markers support the idea that vascular damage leads to myositis (Bae *et al.*, 2022).

In individuals with less severe skin disease, the gut microbial diversity was less varied. The study found that imiquimod-induced skin inflammation was less severe in psoriasis murine models treated with antibiotics to lower gut bacterial

diversity. This implies that decreased gut microbial diversity may change the proinflammatory T cell response, resulting in less severe skin inflammation. In our study, the prevalence of the *Dorea genus* was likewise connected with higher DM skin disease activity. *Dorea* species are frequent inhabitants of the gut microbiome and have been shown to enhance gut permeability and consume host mucus as a source of energy. Given the prominent interferon signatures in DM skin disease, it is interesting that several *Dorea* species, such as *Dorea longicatena* and *Dorea formicigenerans*, have been linked to increased stimulus-induced interferon responses in large population investigations (Bae *et al.*, 2022).

2.3 Molecular mimicry correspond to autoimmune diseases.

There are four criteria addressed in molecular mimicry, such as identical properties between epitope of host and epitope of microorganisms or foreign antigens, presence of T cells which cross-react with both epitope, exposure of microbe or foreign antigens, and autoimmunity caused by epitopes from either infection of microbes or other environmental materials. The infection of *Campylobacter jejuni* cause Guillain Barré Syndrome (GBS) and association of butyrophilin, a bovine milk protein in multiple sclerosis (MS) showed the relationship of molecular mimicry in autoimmune diseases (Rojas *et al.*, 2018).

The rheumatoid arthritis (RA) majorly caused by microbe infections, such as *Porphyromonas gingivalis*, *Proteus mirabilis*, *Escherichia coli*, and Epstein-Barr virus (EBV). Compare to all the microbes, *P. gingivalis* is the one major cause of RA which is also cause of periodontal disease. Several studies showed that enolase in *P.gingivalis* and α-enolase in human have similar structure about 82%. Thus, antibodies produced by immune system against enolase of *P.gingivalis* recognise as

self-antigen and promoting cross-reactivity which lead to autoimmunity (Rojas *et al.*, 2018).

Antiphospholipid syndrome (APS) is closely related to SLE and is occasionally a consequence of SLE. Anti- ß$_2$-GP1 antibody is a defining feature of APS and a diagnostic characteristic for SLE, which has a bad prognosis. The ß$_2$-GP1 ortholog *of E. gallinarum* is implicated in producing anti- ß$_2$-GP1 antibodies. *Roseburia intestinalis*, a gut commensal that carries ß$_2$-GP1 mimotopes, is likewise implicated in an APS model for cross-activating T/B cells, eliciting pathogenic autoimmune responses, and causing thrombotic events similar to clinical APS. Anti-R. intestinalis core sequence antibodies were shown to be elevated in APS patients and were linked to anti-ß2GP1 antibodies, which can cross-react with *R. intestinalis*. *R. gnavus* conserved protease-resistant bands have been shown to cross-react with nephrotoxic anti-dsDNA antibodies. Indeed, antibody levels against *R. gnavus* strain CC55_001c were favourably connected with SLEDAI and anti-DNA antibody levels, but negatively correlated with C3, making this strain a possible measure for lupus disease activity. (Li *et al.*, 2021).

According to studies, human Ro60 causes *Propionibacterium propionicum*-reactive T cells to multiply dramatically. In GF C57BL/6 mice monocolonized with *Bacteroides thetaiotaomicron* harbouring Ro60 ortholog, anti-human Ro60 IgG may be found. The homology between human Ro60 and microbial peptides leads to the conclusion that molecular mimicry occurs in both trials. It's interesting to note that the oral bacteria *Lautropia mirabilis* likewise has a main protein sequence that is quite similar to human's Ro60. Impaired gut barrier makes it easier for gut pathobionts to enter internal organs. Mimotope carriers, such as *B. thetaiotaomicron* and *E.*

gallinarum, which both contain the ß$_2$-GP1 mimotope, as well as Ro60 ortholog carriers, can trigger autoimmune reactions by activating bystander cells and autoreactive T/B cells (Figure 2.8) (Li *et al.*, 2021).

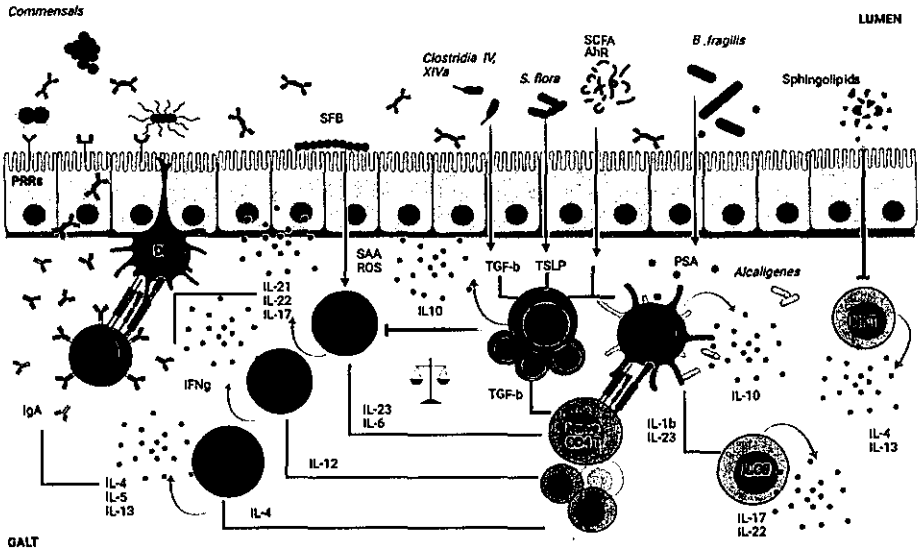

Figure 2.8. Crosstalk between microbiota and T cells in the sustaining of gut homeostasis. Commensal bacteria can induce antigen-specific CD4+T cell reactions via dendritic cells (DC) and/or pattern recognition receptors (PPRs) on enterocytes (Garabatos and Santamaria, 2022).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents that were used in this study are listed in Table 3.1.

Table 3.1. List of general chemicals and reagents.

Chemicals and reagents	Manufacturer/ Brand
1% Agarose gel	Thermo Scientific™
Tris-acetate-EDTA (TAE)	Sigma – Aldrich
Primers 515F and 806R	Illumina Inc.
TaKaRa Ex Taq DNA polymerase	Takara Bio USA, Inc.
0.25% Bromophenol blue	Sigma – Aldrich

3.1.2 Kits and consumables

The kits and consumables that were used in this study are listed in Table 3.2.

Table 3.2. List of kits and consumables.

Kits and consumables	Manufacturer/ Brand
IDT xGen DNA Library Prep EZ Kit	Integrated DNA Technologies
QIA amp PowerFecal Pro DNA Kits	Qiagen

3.1.3 Laboratory apparatus and equipments

The laboratory apparatus and equipments that were used in this study are listed in Table 3.3.

Table 3.3. List of general laboratory apparatus and equipments.

Laboratory apparatus and equipments	Manufacturer/ Brand
White filter membrane	Cytiva
Micropipette	Eppendorf Research®
PCR Tube	Thermowell®
Vortex	Stuart™
Flask	Pyrex

3.1.4 Computer application programmes and softwares

All computer application programmes and softwares that were used in this study are listed in Table 3.4.

Table 3.4. List of computer application programmes and softwares.

Computer application programmes and softwares	Manufacturer/ Version
Basic Local Alignment Search Tool (BLAST)	Version 2.14.1
MicrobiomeAnalyst	Version 2.0

3.1.5 Instruments

The instruments that were used in this study are listed in Table 3.5.

Table 3.5. List of instruments.

Laboratory apparatus and equipments	Manufacturer/ Brand
Thermocycler	Bio-Rad Laboratories, Inc.
Agarose gel electrophoresis	Bio-Rad Laboratories, Inc.