The role of the microbiome in rheumatoid arthritis

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Abstract

The human microbiome plays a vital role in both health and disease. The evolution of molecular techniques to characterise entire microbiome communities has renewed interest in the involvement of microorganisms in the pathogenesis of Rheumatoid arthritis (RA). In this thesis, 16S and ITS amplicon sequencing were used to characterise bacterial and fungal DNA present in a range of human and mouse samples. Firstly, characterisation of the microbiome present in blood samples obtained from human RA, ankylosing spondylitis, and psoriatic arthritis patients was carried out, relative to healthy controls. Results revealed that the bacterial population in the serum of RA patients was distinct from the healthy state. Through the analysis of paired RA patient blood taken before and three months after treatment, partial microbiome normalisation was identified and was particularly evident in seronegative arthritis patients. Next, the presence and identity of bacterial and fungal communities were investigated in samples of synovial fluid obtained from human RA patients and healthy controls. Our findings revealed that the synovial fluid microbiome of RA could be distinguished from control.

Further, IL6, IL71A, IL22, IL23 were elevated in the blood and synovial fluid of RA subjects. The association of IL6 with bacteria and fungi microbiome was observed in the RA synovial fluid. Finally, a characterisation of the bacterial community members presents in the stool, urine, synovial fluid, blood, and serum from collagen-induced arthritis (CIA) and control mouse samples were undertaken. Here, we demonstrated that the bacterial community in CIA stool samples was distinct from the control.

These data propose that the human blood and synovial fluid microbiome and gut microbiome of the mice is modulated by disease status (RA) and therefore have the

potential to serve as a novel biomarker in RA pathogenesis and treatment response. Further, studies are required to investigate these initial findings.

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LIST OF ABBREVIATIONS

ACPA Antibodies to citrullinated protein antigen

ACR American College of Rheumatology

AKA Anti-keratin antibodies

AMP Antimicrobial peptide

APC Antigen-presenting cell

APF Antiperinuclear factor

AS Ankylosing Spondylitis

Bp Base pairs

CD Cluster of Differentiation

SCW Streptococcal cell wall arthritis

CIA Collagen-induced arthritis

CII Collage type II

COX cyclooxygenase

CRP C-reactive protein

dH2O Distilled water

DMARD Disease-modifying anti-rheumatic drug

DNA deoxyribonucleic acid

dsDNA Double stranded DNA

ESR Erythrocyte Sedimentation Rate

EULAR European League against Rheumatism

FC Fragment Crystallizable region

FDR False rate of discovery

FLS Fibroblast-like synoviocytes

FMT Fecal Microbiota Transplantation

GF Germ-Free

GM-CSF Granulocyte-macrophage colony-stimulating factor

HCQ Hydroxy-chloroquine

HLA Human leukocyte antigen

ICAM-1 Intracellular adhesion molecule 1

IFN Interferon

Ig Immunoglobulin

IL Interleukin

ITS2 internal transcribed spacer 2

LPS Lipopolysaccharide

LRC lymphoid tissue-resident commensal

MAIT Mucosal-associated invariant T cells

MAMPs Microbial Associated Molecular Patterns

MHC Major Histocompatibility Complex

MR1 Major Histocompatibility Complex Class I-related gene protein

MTX Methotrexate

MYD88 Myeloid differentiation primary response 88

MΦ Macrophage

NK Cells Natural killer cells

NKT Natural killer T cells

NOD Non-Obese Diabetic

NSAID Non-steroidal anti-inflammatory drug

OA Osteoarthritis

PA Psoriatic Arthritis

PAD Peptidyl arginine deiminase

PADI4 Peptidyl arginine deiminase 4 gene

PAMP Pathogen associated molecular patterns

PBS Phosphate buffered saline

PCoA Principal Coordinates Analysis

PCR Polymerase chain reaction

PD Periodontal Disease

PD-1 Programmed cell death protein 1

PRR Pattern recognition receptors

PTPN22 Protein tyrosine phosphatase non-receptor type 22

QIIME Quantitative Insights into Microbial Ecology

RA Rheumatoid arthritis

RA V0 treatment-naive patients with RA

RA V3 RA patients after three months of treatment

RANK Receptor activator of nuclear factor kappa B

RANKL Receptor activator of nuclear factor kappa B ligand

RF Rheumatoid factor

RNA Ribonucleic acid

rRNA Ribosomal ribonucleic acid

SCFAs Short-chain fatty acids

SCW streptococcal cell wall arthritis

SD Standard Deviation

SE Shared epitope

SFB Segmented Filamentous Bacteria

SPF Specific-pathogen-free

SSZ sulphasalazine

STAT4 Signal transducer and activator of transcription 4

TGF-β Transforming growth factor-beta

Th Cells T helper cells

TLR Toll-like receptor

TNF Tumour necrosis factor

Tph T peripheral helper

TRAF1 TNF receptor-associated factor 1

Tregs Regulatory T cells

UV Ultraviolet

V4 Hypervariable region 4 of the 16S rRNA gene

VEGF Vascular endothelial growth factor

XT Nextera transposase

ZAP-70 Zeta-chain-associated protein kinase

List of papers

1- Hammad, D.B.M., Liyanapathirana, V. and Tonge, D.P., 2019. Molecular characterisation of the synovial fluid microbiome in rheumatoid arthritis patients and healthy control subjects. *PloS one*, *14*(11).

2- Hammad, D., Hider, S.L., Liyanapathirana, V.C. and Tonge, D.P., 2020. Molecular Characterisation of Circulating Microbiome Signatures in Rheumatoid Arthritis. *Frontiers in Cellular and Infection Microbiology*, *9*, p.440.

Chapter 1

1 Literature Review

1.1 Introduction

Rheumatoid arthritis (RA) is a common inflammatory condition, the pathogenesis of which remains unclear. The microbiota has been implicated in the aetiology of rheumatoid arthritis with the first reports dating in the 19th century when Wohlmann and Bannatyne proposed the presence of *Mycobacteria* in the joints and suggested that these may be responsible for the inflammation observed (Yeoh *et al.*, 2013). The development of molecular techniques to characterise entire microbiome communities has renewed interest in the involvement of microorganisms in the pathogenesis of RA.

By the end of 2007, the Human Microbiome Project (HMP) was initiated through the National Institutes of Health (NIH) to obtain a better comprehension of the sophisticated biological interactions between commensal microorganisms and the human body. One such way is via utilising revolutionary culture-independent technology, such as 16S rRNA gene sequencing (Gevers *et al.*, 2012). The specialists in the HMP set out to accomplish two key points:

1) Determination of the microbial populations which are found in different sites of the human body such as the gut, oral, and others.
2) Investigation of the role of the microbiome in human health and illness (Scher and Abramson, 2011).

In this literature review, the pathogenic mechanisms behind RA will be described, and the role of the microbiome in the development and progression of RA will be discussed.

1.2 Rheumatoid Arthritis

1.2.1 Definition, symptoms, epidemiology, and aetiology

RA is one of the most common chronic inflammatory disorders. It initially impacts the joints and is manifested in pain, stiffness, synovial membrane inflammation, hyperplasia of the synovial lining and overactivation of osteoclasts, followed by the destruction of joint bone and cartilage (Liu, 2018) **(Figure 1-1)**.

It is a systemic disease affecting not only the joints but other organs as well, most often the lung, pleura, pericardium, and skin (Liu, 2018). The signs of RA are the systemic loss of regulation of immune system distinguished via either acute or chronic inflammatory response, in which the immune system mainly attacks the joints of the body leading to tissue pathology and clinical illness (Liu, 2018).

It impacts approximately 1% of the world's population. Around 400,000 people in the United Kingdom have RA, and about 58 million people suffer from RA all over the world. Globally, the highest prevalence rates are seen in peoples of the Pima Native Americans, where prevalence is up to 10 times higher than those of most population groups have been identified. The disease affects both sexes, and to be twice as common in women (Liu, 2018).

The mortality rates of arthritic patients are higher than the healthy population (Beirith, Ikino and Pereira, 2013). There are three notable factors as to why RA patients die prematurely, as well as evidence to suggest the reasons behind the expansion of the mortality gap between RA and healthy patients. The first is the correlation between immune dysfunction and systemic inflammation with RA, as these seem to accelerate and promote mortality

(Gabriel, 2008). The second is that RA patients do not seem to receive optimum health care and preventive care (Gabriel, 2008). The third is that RA patients have a higher risk of different comorbid severe conditions and tend to experience worse results following the incidence of these diseases (Gabriel, 2008).

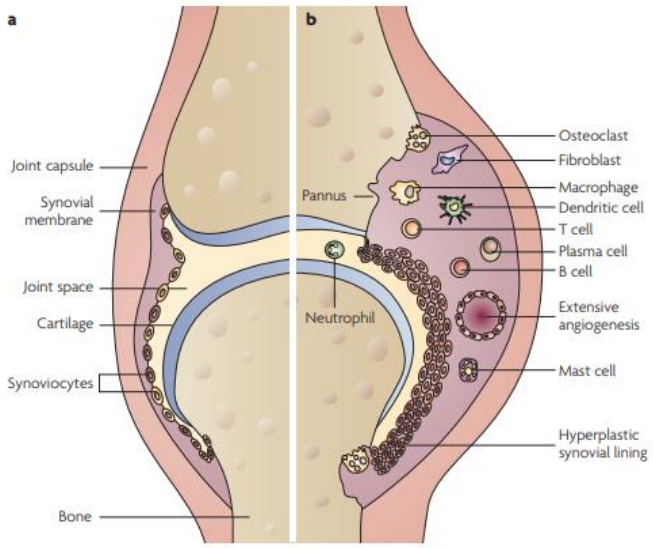

Figure 1-1 Schematic illustration of a healthy joint and RA joint. A normal joint (a) in comparison to a joint influenced with RA (b) with classical characteristics of the condition: joint location narrowing, hyperplasia and immune cell infiltration of the synovial membrane (Strand, Kimberly and Isaacs, 2007).

Its aetiology is not entirely understood, but genetics and microbial dysbiosis factors both contribute. The fundamental cause of RA is inherited factors, which is credited for approximately 50 per cent of the risk components for RA (McInnes and Schett, 2011). Twin investigations are a source of proof of this, and there have been investigations that demonstrated the increased rates of RA among identical twins, approximately from 15% to 30%, while the rate of RA in fraternal twins is significantly lower at 5% (McInnes and Schett, 2011). This has been credited to the presence of the

Human Leukocyte Antigen (HLA) genotype [also known major histocompatibility complex (MHC)] in RA patients. The HLA system encodes membrane proteins on antigen-presenting cells (APC) that display antigen peptides to T lymphocytes, and initiate the adaptive immune response. The HLA genes can be classified into three classes (IIII), where class I and II are involved in cell-mediated immune responses. In contrast, class III genes encode proteins regulating immune responses, such as tumour necrosis factor (TNF), complement proteins (C2, C4), and heat shock proteins (Smolen *et al.*, 2007). Notably, the HLA genotype is associated with the development of RA, such as the HLA-DBR1 (alleles contain a common sequence of amino acids at position 70-74 of the HLA-DRβ chain (QKRRA or QRRAA or RRRAA) within the third hypervariable region of the HLA-DRB1 molecule, which considers a part pivotally essential for peptide binding between antigen-presenting cells such as dendritic cells and T cells) (Smolen *et al.*, 2007; Actor and Actor, 2012). Another example of the effect of hereditary components in RA patients is peptidyl arginine deiminase type IV (PADI4). This is a human protein which encodes enzymes responsible for the alteration of arginine to citrulline residues. Other examples include polymorphisms in protein tyrosine phosphatase N22 (PTPN22), a human protein which is found primarily in lymphoid tissues and is involved in a number of signalling pathways related to the immune response, and activator of transcription 4 (STAT4), a protein which is associated with IFN-γ production in response to IL-12 and the evolution of Th1 cells from naive CD4+ T cells (Bacon *et al.*, 1995; Cloutier and Veillette, 1999; Suzuki *et al.*, 2003; Kaplan, 2005; Choy, 2012).

Of interest to this research is another factor that causes RA: microbial dysbiosis. This has been proposed to cause RA in many research studies (QuanQiu Wang and Xu, 2019), but the proof is not yet decisive. The assumption that microorganisms cause RA has been circulating for more than seven decades (Scher and Abramson, 2011), yet the evidence is

lacking. It is suggested that microbiota can be shown to affect arthritic patients directly and that these microorganisms have virulence agents which allow the initiation of an immune response in joints (Rajappan, Joseph and Paul, 2015).

1.2.2 **Pathophysiology and mechanisms underlying disease process**

RA aetiology is complex due to the high number of human cells involved in RA pathogenesis. This includes the innate and adaptive immune cells, and resident cells of the joints, such as dendritic cells, macrophages, T cells, and B cells, play vital roles in RA pathogenesis. These cells can either reside in the blood or synovium (Lubberts, 2010). Furthermore, there are a number of pro-inflammatory cytokines implicated in the pathogenesis of RA, such as interleukin-1 (IL-1), tumour necrosis factor α (TNFα), and interleukin-6 (IL-6), which all function to stimulate the inflammatory response in the synovial tissue (Lundy *et al.*, 2007; Lubberts, 2010).

It is thought that an autoantigen such as microbial DNA is taken up by antigen-presenting cells, prototypically dendritic cells, leading to stimulation of the innate immune system, which includes IL-6, IL-23, and transforming growth factor-beta production and also T cells (and thus adding the adaptive immune response) through respective antigen presentation, and costimulation (Riedhammer and Weissert, 2015). The involvement of the shared epitope SE) (a 5-aa sequence motif in the third allelic hypervariable region of the HLA-DRβ chain) proposed that either particularly arthritogenic peptides bind with high affinity to these but not another HLA molecules or that an arthritogenic T cell repertoire is selected by the shared epitope (Korn *et al.*, 2009). These activated T cells, formerly believed to be Th1 (gamma interferon production) cells, are currently thought to belong to the Th17 family (IL-

17 production) and insufficiently controlled by regulatory T cells in RA patients (Korn *et al.*, 2009). They, in turn, stimulate macrophages and produce B cell help (Korn *et al.*, 2009). These events presumably happen partly centrally and partially within the synovial membrane in which the cells have moved. Stimulated macrophages produce pro-inflammatory cytokines such as IL-1, IL-6 and TNF alpha (Derksen, Huizinga and van der Woude, 2017). Activated B cells in RA generate autoantibodies which, after forming immune complexes, which bind to Fc- and complement receptors and consequently increase macrophage cytokine-producing (Derksen, Huizinga, and van der Woude, 2017). In parallel, fibroblast-like synovial cells (FLS) become stimulated and generate inflammatory mediators (Yap *et al.*, 2018). Indeed, fibroblast-like synovial cells may represent an important role, since (1) these cells may "travel" into the blood from one joint to other joints, thus spreading RA (Lefèvre *et al.*, 2009), and (2) mesenchymal overexpression of TNF is adequate to drive all aspects of destructive arthritis (Blüml *et al.*, 2010).

Taken collectively, all these events cause the inflammation of the synovial. The inflammatory response is a result of the production of pro-inflammatory cytokines such as IL-6 and TNF, as evidenced by clinical trials and therapies that target IL-6 and TNF (Mackay and Rose, 2014). Additionally, IL-1 has been observed to play a secondary role. Consequently, other mediators of inflammation, such as small molecules (like prostaglandins), various chemokines, metalloproteinases, are produced and increase the inflammatory response, which clinically present as localised joint swelling and pain (Mackay and Rose, 2014).

Through whichever way the detailed events result (i.e., whether the innate immune reactivity, T or B cell stimulation are predominant), the mechanisms eventually lead to an increase of inflammatory cells inside the synovial membrane; the inflamed synovial

membrane transforms into an autonomous "semi malignant" tissue (pannus) leading to damage of bone and cartilage (Mackay and Rose, 2014). Bone damage is mediated via osteoclasts activated inside the synovial membrane at places adjacent to bone. While osteoclast cell differentiation and activation are pivotally reliant on, pro-inflammatory cytokines induce the production and activity of osteoclasts, and also receptor activator of NFκB (RANK) and its ligand (RANKL)(Mackay and Rose, 2014). Further, cartilage destruction seems to occur mainly by the direct action of metalloproteinases excreted inside the joint on the cartilage matrix or through the activation of chondrocytes via cytokines and following matrix degradation (Mackay and Rose, 2014). The hypothesis is predicted for the mechanism of RA is shown in **Figure 1-2**.

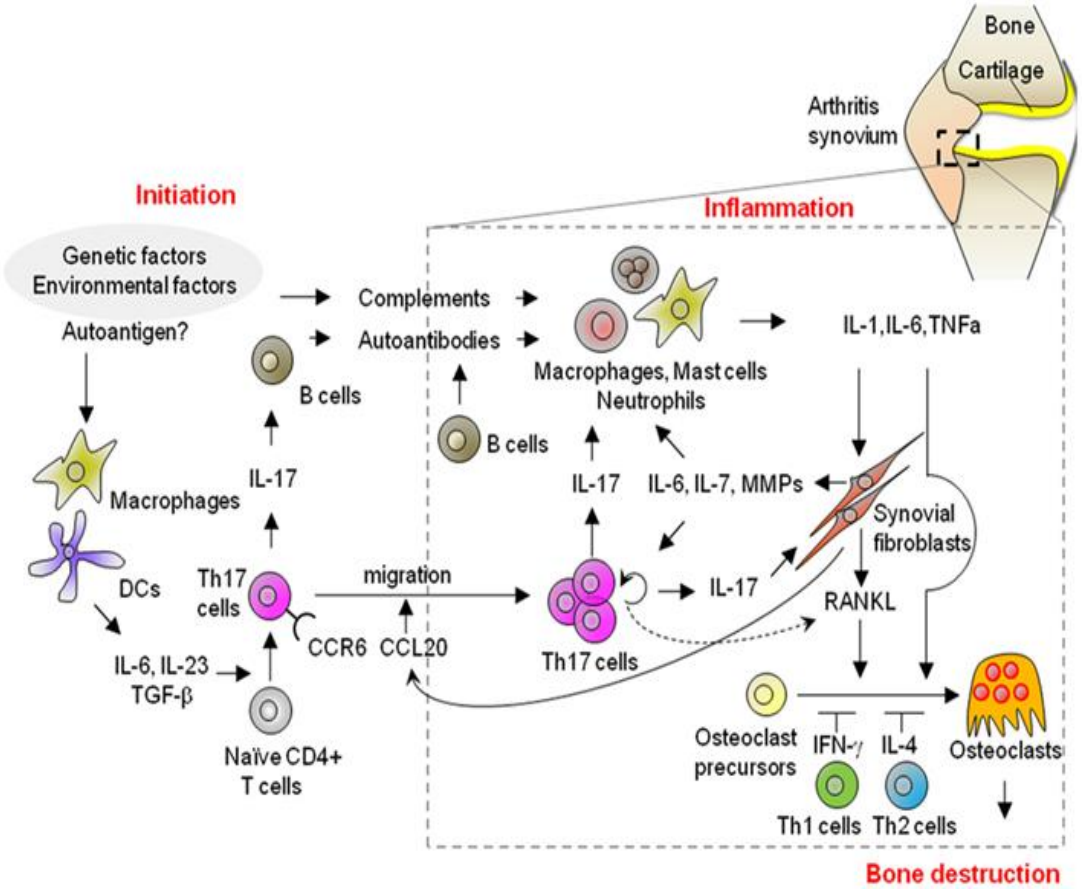

Figure 1-2 Hypothesis is predicted for the mechanism of RA. Several resident cells of the human body participate in the RA, such as innate immune cells as (dendritic cells and macrophages), and also resident cells of the joints, for example, such as chondrocytes and fibrocytes cells. Furthermore, pro-inflammatory cytokines (tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6) function in the pathogenesis of RA. The main T cell class in RA is TH17 that participate in the evolution of RA through secreting IL-17, which stimulate the bone damage by activating osteoclast cells with receptor activator of nuclear factor kappa-B ligand (RANKL) (RANKL binds to RANK expressed by osteoclasts and osteoclast precursors to stimulate osteoclast differentiation) resulting in bone erosion. Moreover, this interleukin activates several cells like macrophages and fibroblast cells to bring about inflammation of joints. Furthermore, IL-17 stimulates macrophages and chondrocytes to damage of the cartilage. Additionally, it motivates B cells to produce autoantibodies, such as a rheumatoid factor (Komatsu and Takayanagi, 2012).

1.2.3 **Classification of Rheumatoid Arthritis**

Clinical signs of RA vary depending on the involved joints and the condition stage. In order to facilitate the consistent identification of patients, classification criteria have been developed. **Table 1.1** summaries the 1987 American Rheumatism Association revised criteria for RA classification. Until recently, RA diagnosis should have four or more of these criteria and must be present for at least six weeks to exclude another differential diagnosis of arthritis such as osteoarthritis and connective tissue arthritis (Arnett *et al.*, 1988).

Although these criteria present the gold standard for condition definition, they may have significant limitations in allowing earlier RA classification. Therefore, a joint working group have updated these criteria; the 2010 American College of Rheumatology /European League Against Rheumatism (2010 ACR/EULAR) criteria are designed to aid the identification of patients who would benefit from early intervention, as presented in **Table 1.2** (Villeneuve, Nam, and Emery, 2010). A patient with synovitis, not explained by another disorder, and which meets these initial criteria with a score of ≥6/10 can be classified as having "definite RA."

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Table 1-1 1987 American College of Rheumatology Revised Criteria for rheumatoid arthritis adapted from (Arnett *et al.*, 1988)

Criteria	Definition
Morning stiffness	Morning stiffness ≥ 1 hour for at least 6 weeks
Arthritis ≥ 3 joints	Synovitis in ≥ 3 joint areas simultaneously
Arthritis of hand and wrist	Wrist or hand MCP or PIP joints for at least 6 weeks
Symmetric arthritis	Same joint areas on both sides of the body, for at least 6 weeks
Rheumatoid nodules	Non tender subcutaneous nodules situated over bony prominences
Serum rheumatoid factor	IgM Autoantibody directed against the Fc fragment of IgG
Radiographic changes	Typical of rheumatoid arthritis soft tissue swelling and juxta-articular demineralization, uniform joint space narrowing and erosion

Four of seven criteria are needed for diagnosis of RA and the 14 possible joint areas include hand proximal inter-phalangeal (PIP) and metacarpal phalangeal (MCP), wrist, elbow, knee, ankle, and foot metatarsal phalangeal (MTP) on either side for at least 6 weeks.

Table 1-2 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis adapted from Villeneuve, Nam, and Emery, 2010.

Criteria	Score
Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
Serology (at least 1 test result is needed for classification)	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Score of ≥6/10 is needed for classification of a patient as having definite RA. Joint involvement refers to any swollen or tender joint during examination and is also classified according to location and number of involved joints. Serology, low positive and high positive are equivalent to ≤3 and >3 times the upper limit of normal, respectively and are based on the reference range of the laboratory that assesses the biomarker.

RF = rheumatoid factor; ACPA = anti-citrullinated protein; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate

1.2.4 **Diagnosis of Rheumatoid Arthritis, therapy, and treatment**

RA diagnosis depends mainly on the history of patients, imaging such as x-ray and laboratory techniques such as serological tests (Sommer *et al.*, 2005). For example, antibodies appearance, such as the antibody RF (most generally immunoglobulin M directed versus the constant fragment section of the immunoglobulin G protein).

Further to RF, a variety of other autoantibodies have been historically correlated with RA. Of these, the most significant were antibodies binding to keratin (anti-keratin antibodies), and the so-called anti-perinuclear factor (APF) (NIENHUIS and MANDEMA, 1964), later identified as antibodies to filaggrin, a keratin-binding structural protein (Sebbag *et al.*, 1995). As filaggrin is not expressed in the joint, further research into the valid antigenic target of these autoantibodies eventually led to the historic discovery that their real goal was, in fact, citrullinated proteins (Schellekens *et al.*, 1998; Girbal-Neuhauser *et al.*, 1999).

These autoantibodies are now collectively referred to as anti-citrullinated protein antibodies (ACPAs) or also known as anti-CCP. The cyclic citrullinated peptide (CCP) test, which was the first commercially available assay for ACPA positivity, was based on cyclic citrullinated filaggrin-derived peptides (Schellekens *et al.*, 2000). However, the second generation CCP test (CCP2) currently used in many clinics use a combination of synthetic citrullinated peptide epitopes derived from a phage display library to provide an optimal combination of sensitivity and specificity for diagnosing RA (Van Venrooij and Zendman, 2008). Notably, the CCP2 peptide does not contain sequences derived from human proteins.

Serological surveys have noted that 69% of arthritis patients are positive for RF, and 67% of these patients carry ANTI-CCP (Shmerling and Delbanco, 1991; Lindqvist *et al.*, 2005; Coenen *et al.*, 2007). There are other laboratory tests such as complete blood tests, C-

reactive protein, and erythrocyte sedimentation rate that helps to obtain an indicator for an early stage of RA (SOX and LIANG, 1986).

Much research has gone into not only to diagnose RA early but also to produce therapy that will enable the (1) reduction of pain, (2) decrease of the inflammatory response in the joints, (3) inhibit or delay of joint destruction, and (4) minimise disability of patients, maximising their mobility and ability to lead a healthy life. RA treatments such as analgesics (painkillers such as ibuprofen or naproxen) and non-steroidal anti-inflammatory drugs (NSAIDs) are used to reduce pain and stiffness of joints. NSAIDs act on blocking an enzyme called cyclooxygenase (COX) from making hormone-like chemicals, which are called prostaglandins. Prostaglandins are one of the body's most significant contributors to inflammation (Dixit, Bhardwaj, and Sharma, 2012).

Furthermore, disease-modifying antirheumatic drugs (DMARDs) assist in lessening inflammation and pain of joints and in inhibiting continuous joint destruction. DMARDs are commonly utilised in the treatment of RA and can be classified into two groups:

1) Conventional DMARDs such as methotrexate (MTX) remains the standard first-line DMARD in RA, which inhibits the enzymes involved in purine metabolism, leading to the increase of adenosine, which causes inhibition of T cells and B cells activation and suppression of intercellular adhesion molecule expression by T cells (Smolen *et al.*, 2020). Also, MTX works to inhibit the binding of IL 1-beta to its cell surface receptor. Other commonly used conventional DMARDS are leflunomide, which inhibits the reproduction of rapidly dividing cells, especially lymphocytes , via inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which plays a vital role in the *de novo* synthesis of uridine monophosphate (rUMP), which is required for the synthesis of DNA and RNA, hydroxy-chloroquine that reduces the activation of dendritic cells and the

inflammatory process by decreasing toll-like receptor signalling. Sulfasalazine has antibacterial, immunosuppressive, and anti-inflammatory effects (Smolen *et al.*, 2014, 2020).

2) Biological DMARDs primarily targeting inflammatory cytokines, the most common of these have been biologicals inhibiting anti-tumour necrosis factor (TNF), including adalimumab, Infliximab, etanercept, certolizumab, and golimumab (Smolen *et al.*, 2020). In the patient's refractory to anti-TNF, B-cell reduction through the anti-CD20 antibody rituximab can be useful. Other biologicals include tocilizumab, which targets the Interleukin-6 receptor, Anakinra, which targets the IL-1 receptor (Smolen *et al.*, 2014, 2020).

Recently, there is a combination of two types of DMARDs to RA treatment. For example, using one of the conventional DMARDs as Methotrexate with others from biological DMARDs, for example, rituximab to RA therapy (Smolen *et al.*, 2014, 2020). Such examples are many and aim to reduce symptoms of RA so that the patient can lead a healthy life.

1.2.5 Ankylosing spondylitis, and psoriatic arthritis

Ankylosing spondylitis and psoriatic arthritis belong to a group of inflammatory diseases known as the spondyloarthritides (Au et al., 2017). Ankylosing spondylitis (AS) is a persistent autoimmune condition distinguished via the inflammation of the peripheral joints, the axial skeleton, and the attachments of enthuses and ligaments (C. et al., 2017). RA and ankylosing spondylitis are chronic and progressive inflammatory joint diseases that

lead to joint destruction and functional disability (Barczyńska *et al.*, 2015). The clinical signs of RA and ankylosing spondylitis differ in several ways. The age of onset in RA is much older, with an average beginning of 40–50 years compared with 28 years in ankylosing spondylitis, and with a female predominance (3:1) compared with the male dominance in ankylosing spondylitis (van der Horst-Bruinsma *et al.*, 2009; Barczyńska *et al.*, 2015). The genetic association with HLA alleles is more effective in AS, with an HLA-B27 antigen in 95% of the patients in comparison to RA, with 60% HLA DR1 or DR4 positives (van der Horst-Bruinsma *et al.*, 2009; Azevedo and Buiar, 2013). The type and localisation of AS, the arthritis is mainly localised in the spine, and sacroiliac joints with an oligoarthritis of the larger joints (hips, knees, shoulders), whereas in RA is peripheral polyarthritis in RA, particularly with the involvement of feet and hands. Radiographic imaging of RA patients identifies bone resorption with erosive alterations in contrast with an ankylosing disease where bone formation with vertebral syndesmophytes is present (van der Horst-Bruinsma *et al.*, 2009). Extra-articular manifestations can occur in both conditions, but again these manifestations differ in the heart (pericarditis in RA, conduction disturbances in AS), the eye (keratoconjunctivitis sicca and scleritis in RA, versus anterior uveitis in AS), lungs (pleural lesions or nodules in RA and fibrosis in AS) and gut (peptic ulcers in RA and colitis in AS) (van der Horst-Bruinsma *et al.*, 2009). Both conditions respond well to therapy with NSAIDs; however, DMARDs, which are very significant in RA, have limited value in AS. TNF alfa blocking drugs, but, found a high efficacy in both conditions (van der Horst-Bruinsma *et al.*, 2009).

Psoriatic arthritis (PA) is a persistent inflammatory joint disorder observed strongly combined with chronic inflammatory skin condition psoriasis (Gilis et al., 2018). Both RA and psoriatic arthritis are prevalent, persistent inflammatory conditions; both are distinguished via pain and swelling in the joints and have significant systemic

manifestations (Villeneuve *et al.*, 2013; Verheul *et al.*, 2015; Zabotti *et al.*, 2016). If not diagnosed and treated early, both can lead to joint damage with loss of function. For this reason, early diagnosis is essential to determine curative strategies that will optimise clinical and radiographic outcomes(Gladman, 2015).

RA and psoriatic arthritis have critical differences in radiographic findings, clinical presentation, comorbidities, and pathogenesis to differentiate between these common forms of chronic inflammatory arthritis (Merola, Espinoza and Fleischmann, 2018). Joint involvement is typically, but not always, asymmetric in psoriatic arthritis, while it is predominantly symmetric in RA (Merola, Espinoza and Fleischmann, 2018). Cervical spine involvements, and bone erosions, without new bone growth, are characteristic of RA, while axial spine involvement, nail dystrophy, and psoriasis are distinctive of psoriatic arthritis (Merola, Espinoza and Fleischmann, 2018). Psoriatic arthritis patients typically have seronegative test results for cyclic citrullinated peptide antibodies and rheumatoid factor, while roughly 80% of RA patients have positive findings for CCP and RF antibodies (Merola, Espinoza and Fleischmann, 2018).

1.3 Immune cells

As an autoimmune illness, dendritic cells, macrophages, T cells, and B cells play crucial roles in the pathogenesis of RA. These cells can either exist in the synovium or peripheral blood. Dendritic cells, along with macrophages and B cells, can present antigen to T cells, and therefore perform a pivotal role in the evolution of innate and adaptive immune responses (Yap *et al.*, 2018). Activated macrophages secrete a variety of cytokines and chemokines to maintain the inflammation in the joints. In RA, the central function of T cells is to stimulate macrophages and fibroblasts and modify them into tissue-destructive cells (Yap *et al.*, 2018). However, B cells secrete physiologically critical proteins such as RF, ACPA, and pro-inflammatory cytokines that are involved in promoting RA. B cells also mediate T cell activation by the expression of co-stimulatory molecules (Yap *et al.*, 2018). This part provides detailed information on how different immune cells participate in RA pathogenesis.

1.3.1 Innate immune cells

Innate immune cells are the primary barrier to preserving human body homeostasis by tolerating the commensal bacteria and enhancing immunity to pathogenic species (Chen and Kasper, 2014).

One of these cells is dendritic cells, which are located in tissues that are in contact with the external environment, such as the gut, skin, and the inner lining of, nose, and lungs (Rescigno, 2011). They are professional antigen-presenting cells. Their primary role is to process antigen material and present it on the cell surface on the T cells of the immune system. They function as messengers connecting the innate and the adaptive immune

systems (Wculek *et al.*, 2019). Before finding foreign antigen, dendritic cells express low levels of MHC II protein and co-stimulatory molecules on their cell surface. These immature dendritic cells are ineffective at presenting antigen to T cells (Wculek *et al.*, 2019). Once a dendritic cell's pattern-recognition receptors recognise a pathogen-associated molecular pattern, the antigen is phagocytosed, and the dendritic cell then becomes stimulated, upregulating the expression of MHC II protein (Wculek *et al.*, 2019). It additionally upregulates several co-stimulatory molecules required for T cell activation, including CD40 and B7. The latter can interact with CD28 on the surface of a CD4+ T cell (Wculek *et al.*, 2019). The dendritic cell is then a fully mature professional antigen-presenting cell (Wculek *et al.*, 2019).

Plasmacytoid and myeloid dendritic cells represent the two main dendritic cells subsets and can be identified based on their expression of surface markers, morphology, and function (Canavan *et al.*, 2015). They can also be located in an immature state in the blood. Once activated, they move to the lymph nodes where they interact with B cells and T cells to initiate and also shape the adaptive immune response (Sarkar and Fox, 2005). It was reported that the dendritic cells possessed the unique capacity to stimulate naive T lymphocytes through their first contact with an antigen (Pan *et al.*, 2017). According to several studies, dendritic cells play an important role in RA patients, and the primary mechanism is the initiation and maintenance of inflammation via presenting antigens to autoreactive T cell (Sarkar and Fox, 2005; Pan *et al.*, 2017; Yap *et al.*, 2018).

Dendritic cells have been observed in synovium and joint fluid in RA, usually at the centre of a cluster of T cells. These dendritic cells express MHC II, the co-stimulatory molecules CD40, CD80, CD86, adhesion molecules such as dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin and chemokine receptors such as the CC-chemokine receptor 7 (Sarkar and Fox, 2005). Dendritic cells can polarise T cells into Th1

or Th2 phenotypes based on the cytokine environment. Th1 responses are initiated in the context of IL-12 and IL-23. The cytokine milieu of the RA synovium promotes dendritic cell differentiation and role that could lead to autoantigen presentation to T cell (Sarkar and Fox, 2005; Yap *et al.*, 2018).

In the intestine, dendritic cells differentiate a wide range of microbiota by specific sensing receptors, for instance, toll-like receptors (TLRs) (Chabot *et al.*, 2006). Presently there is some evidence to indicate that there are some TLRs subtypes implicated in the disease severity within animal models of rheumatoid arthritis, for instance, TLR4 (Abdollahi-Roodsaz *et al.*, 2012). It should be noted that dendritic cells in an active stage can excrete cytokines and chemokines, which involve IL-6 and IL-23 that are associated with the migration of dendritic cells and inflammation (Wesa and Galy, 2002). Furthermore, dendritic cells in the gut regulate gut-specific IgA output to inhibit microbial contact with the linings of the intestinal epithelial cells (Suzuki *et al.*, 2007).

Macrophages are also thought to be involved in RA pathogenesis. These cells are fundamental cellular components of the innate immune system, together with osteoclasts and myeloid dendritic cells, which are derived from myelomonocytic origins (Gierut, Perlman, and North, 2010). Macrophages distinguish from blood monocytes and have fundamental roles in tissues as phagocytes of attacking pathogens and as scavengers of apoptotic debris (Gierut, Perlman and North, 2010). Activated macrophages have two distinct phenotypes associated with different stimuli: M1 (classically activated) and M2 (alternatively activated) (Laria *et al.*, 2016). M1 macrophages secrete high levels of pro-inflammatory cytokines such as TNF, IL-1β, reactive nitrogen, and oxygen intermediates eliminating microorganisms and tumour cells; however, M2 macrophages are included in the resolution of inflammation by decreased production of pro-inflammatory cytokines, phagocytosis of apoptotic neutrophils, and increased synthesis of mediators critical in

tissue remodelling, angiogenesis, and wound repair (Laria *et al.*, 2016; Siouti and Andreakos, 2019).

Macrophages are consistently present in synovial tissue. Most of these immune cells reside within the tissues in a resting state under normal conditions (Yap *et al.*, 2018). Nevertheless, in an inflamed joint of RA, they control the excretion of pro-inflammatory cytokines and damaging enzymes that are linked to inflammatory responses and consequently leading to joint damage (Raimund W. Kinne *et al.*, 2000; Yap *et al.*, 2018). Other than generating cytokines and enzymes, macrophage also mediates multiple RA-related biological processes such as fibroblast proliferation, recruitment of lymphocytes, angiogenesis, cartilage damage, and joint erosion (Raimund W. Kinne *et al.*, 2000; Yap *et al.*, 2018). Similar to dendritic cells and B cells, macrophages act as antigen-presenting cells. They are found to express high levels of HLA-DR and leukocyte adhesion molecules, which allow macrophage to participate in T cells activation alongside B-cells (Schlegel *et al.*, 2013; Yap *et al.*, 2018). The macrophage-mediated T cell activation results in the generation of effector T-cells and expression of resulting pro-inflammatory mediators such as IL-1α, and IL-1β which enhance the RA pathogenesis (Bondeson *et al.*, 2006; Siouti and Andreakos, 2019).

In the gut, macrophages are found in considerable numbers in the lower gastrointestinal tract (both small and large intestines) and are considered to guard the gastrointestinal systems. The primary function of this type of cell is connecting with stray microorganisms; for example, members of the microbiota that have broken the barrier of epithelial cells (Cruz et al., 2007). The resident microorganisms are phagocytosed and quickly killed by circulating macrophages. These macrophages utilise mechanisms that involve the generation of reactive oxygen species and antimicrobial protein (Cruz et al., 2007). Moreover, gut macrophages function rehabilitation of the epithelial cell boundary after

microbial infection or injury (Reid *et al.*, 2011). Rebuilding this site following injury is essential to prevent microbial infiltration and sepsis in such a microbe-laden environment (Pull *et al.*, 2005). Macrophage stimulation in the inflamed synovium is associated with the severity of arthritic illness (Raimund W Kinne *et al.*, 2000).

1.3.2 **Adaptive immunity**

1.3.2.1 T cells

T cells are one of the main components of the cell-mediated immune response. They are essential in hosting an immune response against pathogens. T cells play a significant role in defence against intracellular pathogens such as intracellular bacteria, and in immunity to extracellular pathogens through activation of B cells and the subsequent production of antibodies(Levinson, 2014; Marshall *et al.*, 2018). The antigen-presenting cells process the antigen and display it as an MHC molecule on its cell surface. T cells then recognise the antigen-MHC complex through T cell receptors and become activated (Marshall *et al.*, 2018). After stimulation, the T cells produce cytokines to directly attack pathogen cells and enable the growth of more T cells. Some T cells will become memory T cells in preparation for future infections, and others become cytotoxic T cells to attack virus-containing cells (Levinson, 2014; Marshall *et al.*, 2018).

Extensive research has been carried out in an attempt to explain the role of T cells in RA, particularly the T-cell activation (Meednu *et al.*, 2016). T cells can be stimulated through different cell types, including dendritic cells, macrophages, and B cells. Although the specific roles of T cells in RA are not entirely understood, increasing evidence suggests that CD4+ T cells are particularly crucial in RA pathogenesis. Through the stimulation of T-

cells, CD4+ T-cells interact with MHC II molecules as well as co-stimulating molecules, for instance, CD28, which is expressed on the surface of antigen-presenting cells (Podojil and Miller, 2009). The role of CD4+ T-cells in the inflammation of RA was established through its connection with the specific MHC-II alleles, HLA-DR4 containing similar amino acid motifs in the third hypervariable region of DRB-chain. This reaction then leads to a more aggressive form of RA (Cope, 2008). Further to cell-to-cell interaction, contemporary pieces of evidence also propose that CD4+ T-helper cells principally participate in the RA pathogenesis by the excretion of chemokines and cytokines such as IL-17, IL-21, IL-22, and IL-23. These cells are significant immune modulators in cell-mediated immunity (Meednu *et al.*, 2016).

T-cell subsets, for instance, type 1 T-helper (Th1) cells, are highly stimulated in RA, and they excrete pro-inflammatory cytokines, for example, IFN-gamma, TNF-α, and IL-2 (Meednu *et al.*, 2016). Besides, Th1 cells stimulate macrophages to function as antigen-presenting cells to present MHC-II molecules to the T cells (Cope, 2008). Other types of CD4+ T-cells are Th17, and regulatory T (Treg) cells perform fundamental roles in the pathogenesis of RA (Alunno *et al.*, 2015). Th17 cells excrete IL-17, which activates the stimulation of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases (Alunno *et al.*, 2015). Th17 cells migrate into synovial tissue and participate in the bone destruction in arthritis through upregulating receptor activator of nuclear factor-kappa-B ligand (RANKL) on synovial fibroblasts as well as inducing local inflammation. Activated Fibroblast-like synoviocytes (FLSs) secrete various inflammatory mediators to recruit and expand different inflammatory immune cells. Researchers on the origin of Th17 cells in inflammation have shed light on the pathogenic conversion of Foxp3+ T cells (Komatsu and Takayanagi, 2018). Th17 cells converted from Foxp3+ T cells (ex Foxp3 Th17 cells) contain the most potent osteoclastogenic T cell subset in inflammatory bone loss. It has

been proposed that osteoclastogenic T cells may have developed originally to stop a local infection in periodontitis via inducing tooth loss. Besides, Th17 cells also participate in the RA pathogenesis through modulating antibody function (Komatsu and Takayanagi, 2018). Antibodies and immune complexes have attracted considerable attention for their direct role in osteoclastogenesis, and a specific T cell subset in joints was observed to be involved in B cell antibody production (Komatsu and Takayanagi, 2018).

In the past, a higher number of Th17 and higher expression levels of IL-17 have been consistently identified in the serum of RA patients' serum in comparison to healthy people (Al-Saadany *et al.*, 2016). Several investigations observed that both serum IL-17 and circulating Th-17 cells are associated with RA illness activity. Of record, mast cells and macrophages are also principal sources of IL-17 as well as to Th-17 cells (Elhewala *et al.*, 2015). IL-17 activates the production of IL-6, IL-8, vascular endothelial growth factor-A (VEGF-A), Matrix metalloproteinase-1, and Matrix metalloproteinase-2 in RA synovial fibroblasts (Gaffen, 2009b). Some studies have revealed that IL-17 participated in synovial neoangiogenesis, pannus growth, and osteoclastogenesis (Gaffen, 2009b).

Another hand, CD4+CD25+ Treg cells mainly inhibit autoimmunity via suppressing autoreactive lymphocytes mediated via transforming growth factor-beta and IL-10 (Cooles, Isaacs, and Anderson, 2013). Nevertheless, Tregs merely function in suppressing the proliferation of effector T cells but failed to suppress pro-inflammatory cytokines such as IL-6 and TNF-α produced from monocytes and activated T-cells (Boissier *et al.*, 2009). The same group also showed that the use of Infliximab, an anti-TNF-α, could return the Treg activity to prevent inflammatory cytokine generation. Treg cells have been consistently found in the blood and synovial fluid of RA patients (Morita *et al.*, 2016). However, the percentage of Treg cells in RA patients relative to healthy people is highly questionable. Similarly, contradictory findings have also been detected in RA patients when Tregs cell

percentage was associated with multiple clinical features, for instance, condition duration, age, sex, CRP, RF, and ESR (Yap *et al.*, 2018).

Another sort of CD4+ type 2 T-helper (Th2) cells play pivotal roles in B-cell stimulation and immunoglobulin (Ig) class switching to IgE, and excretion anti-inflammatory cytokines, instance, IL-4 and IL-5 (Schulze-Koops and Kalden, 2001).

A noteworthily, T-cells also play a crucial role in activating B cell responses and antibody generation, which consequently participates in RA pathogenesis (Rao, 2018). T follicular helper cells are the predominant T cell population that combines with B cells typically within inflamed peripheral tissues and the follicles of secondary lymphoid organs (Rao, 2018). Typically, T follicular helper cells present B cell-helper phenotype through highly expressing B-cell lymphoma 6 protein (Bcl6), C-X-C Motif Chemokine Receptor 5 (CXCR5), chemokine C-X-C motif ligand 13 (CXCL13), IL-21, and Programmed cell death protein 1 (Rao *et al.*, 2017; Rao, 2018). Interestingly, there is a distinct B cell-helper T-cell population, known as T peripheral helper (Tph) cells, which do not express CXCR5 and only express a modest level of Bcl6 in RA synovium. These CXCR5−PD-1hi Tph cells, once activated, excrete CXCL13 to recruit B cells for IL-21 production and help in B cell survival, proliferation, and maturation (Rao, 2018).

Taken together, many T cells and the respective effector pathways contribute to arthritis disorder by primarily mediating the ongoing inflammatory process. Th-1 cells that mainly secrete pro-inflammatory cytokines were thought to be the central cells causing RA. Following the identification of other T cell subtypes and their function in RA, for example, Th-17, Treg cells, and Th-2, as discussed above, it is evident that RA pathogenesis is much more intricate. This improved our understanding of RA and has an essential influence on condition treatment and management.

Intestinal T cells are frequently found in the lamina propria of the gut and are a vital element of the adaptive immune system. Based on activation, naïve CD4+ T cells can distinguish into four fundamental categories: T helper 1 (Th1), T helper 2 (Th2), regulatory T cells, and T helper (Th17) (Wu and Wu, 2012). Their expression of different cytokines differentiates these different CD4+ T cell types and transcription factors (**Figure 1-3)**. The correct balance and regulation of T-cell categories are a critical component in identifying one's health situation. For instance, Th2 cells play a significant part in inhibiting parasitic infections, while Th1 cells are essential for the host response to intracellular pathogens (Wu and Wu, 2012). It is thought that the imbalance of T helper reactions can cause diseases, such as the Th2 reaction has been associated with allergic responses.

On the other hand, the Th1 and Th17 responses have been correlated with chronic inflammatory illnesses. The regulatory T cell is an essential factor of immunological tolerance; its impairment in the function could result in chronic inflammatory illnesses (Wu and Wu, 2012).

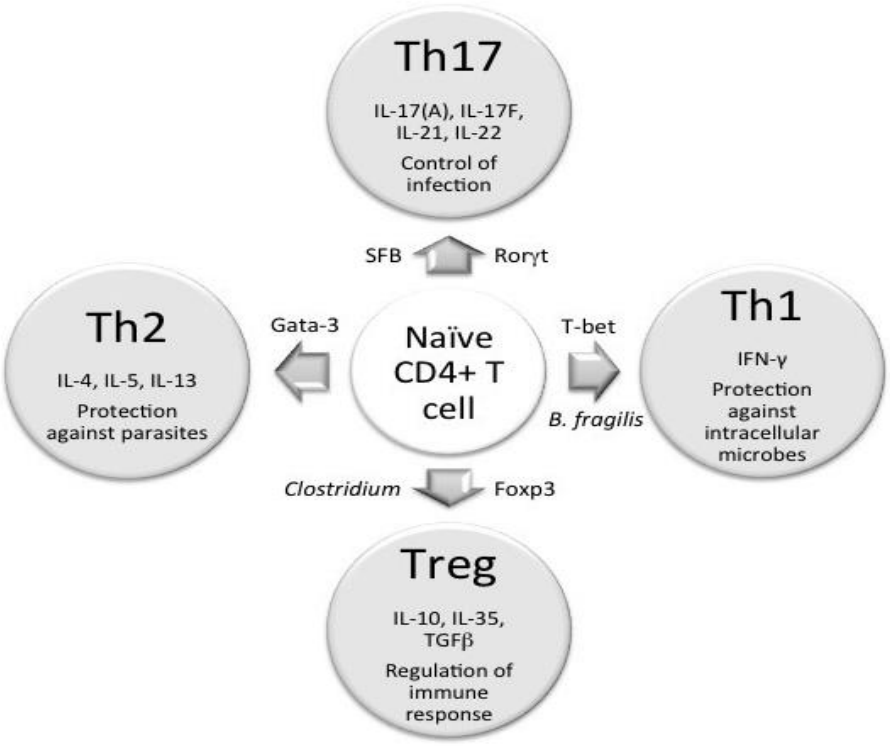


Figure 1-3 Resident bacteria stimulate the diversity of CD4+T cells. Naïve CD4+T cells can distinguish into four main cell sorts: T helper 1 (Th1), T helper 2 (Th2), regulatory T, and T helper (Th17) cells. Once distinguished, each lineage excretes a specific cytokine, as revealed in the image. Th1 cells play a crucial part in removing intracellular bacterial infections; however, the vital role of Th2 is to inhibit parasite infection. Tregs cells play in regulating the immune reaction. Besides, Th17 cells play a fundamental role in regulating infection. Moreover, each subtype differentiation needs the stimulus of a transcription factor that is distinctive to each ancestry. The sort of microbiome classes has been observed to stimulate a specific T cell distinction pathway (Wu and Wu, 2012).

It has been shown that the intestinal microbiota plays a valuable function in the CD4+ T cell maturation, both internal and external, to the gut. Consequently, there is a noteworthy reduction in the quantity of CD4+ cells in the lamina propria and significantly decreased immunoglobulin A in germ-free rats (Macpherson, Geuking, and McCoy, 2005; Chen and Kasper, 2014). Furthermore, GF animals were noted to have an imbalance of Th1/Th2: their immunological reaction is biased to the Th2 response. Moreover, lymph nodes and spleen of germ-free rats show deficiency as well, such as the absence of lymphocyte

zones in these microorganisms (Mazmanian *et al.*, 2005). Recent investigations have also revealed a correlation between particular bacterial sorts with the evolution of specific T-cell species. Segmented filamentous bacteria (SFB) were observed to be a potent inducer of Th17 cells in the lamina propria of the gut (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009a). Ivanov and colleagues demonstrated that the insertion of segmented filamentous bacteria (SFB) into GF rats brought about the elevation of Th17 cells in the intestinal mucosa of the gut (Ivanov *et al.*, 2009a). In the mouse intestine, the existence of SFB has appeared to induce the evolution of Th17 cells, which has a vital role in autoimmune illnesses, for example, arthritis (Wu *et al.*, 2010), experimental autoimmune encephalomyelitis (Lee *et al.*, 2011), and colitis (Stepankova *et al.*, 2007). A current survey elegantly showed the particular labelling and following of intestinal white blood cells. It was demonstrated that these cells move to and from the gut in a normal condition (Morton *et al.*, 2014).

Additionally, intestinal Th17 cells migration in arthritic K/BxN rats was examined and showed that the Th17 cells, which are derived from the gut, terminate in the spleen. The part of the intestine-derived Th17 cells found in the spleen associated with the serum level of autoantibodies that cause autoimmune disease (Morton *et al.*, 2014). This is the primary research that exhibits that the intestinal Th17 cells can participate in autoimmune arthritis. However, there are some investigations proposed that the intestinal microbiota plays a significant part in conserving the stability among pro- and anti-inflammatory T cells, consequently maintaining gut homeostasis. For instance, the introduction of *Bacteroides fragilis* in GF rodents has been found to excite the correct expansion of the immune system. Settlement of mice with *B. fragilis* motivates Tregs and limits a 2, 4, and 6-trinitrobenzene sulfonic acid (TNBS-) growth that stimulates colitis (Round and Mazmanian, 2010). Moreover, another survey presents evidence that the settlement of

mice with microbiota that belongs to the *Clostridium* class also brought about the Tregs activation (Atarashi *et al.*, 2011). Additionally, the establishment of young rats with a mixture of *Clostridium* class brought about defence against dextran sodium sulfate- (DSS-) induced colitis (Atarashi *et al.*, 2011).

Gut CD8+ T cells are predominantly shown in the intestinal mucosa. A low quantity and declined cytotoxicity of these cells in germ-free rats suggest that microbial signals are crucial in preserving the capacity and number of the gut CD8+ T cells (Imaoka *et al.*, 1996; Kawaguchi-Miyashita *et al.*, 1996; Helgeland *et al.*, 2004). These deficiencies may be due to the weakened clonal growth of gut CD8+ cells in murine. Even though not needed for forming the collection of systemic CD8+ T-cell, the intestinal microbiome functions function an imperative part in adopting CD8+ T cells to alter different peripheral blood immune cells, such as plasmacytoid DCs, marginal zone B cells and invariant natural killer T cells (Fujiwara *et al.*, 2008; Wei *et al.*, 2008).

1.3.2.2 B cells

B cells are lymphocytes that are a vital part of the adaptive immune system. They have a protein on the outer surface membrane known as a 'B cell receptor.' This receptor allows B cells to bind to a specific antigen. B cells perform three essential functions: (1) they differentiate into plasma cells and make antibodies against antigens, (2) they play the role of antigen-presenting cells to helper T cells, and (3) they develop into memory B cells after activation by antigen interaction (Gupta *et al.*, 2017).B cells play a significant role in the RA pathogenesis. They express RF and ACPA, two proteins that have been demonstrated to participate in immune complex production and complement stimulation in the joints

(Silverman and Carson, 2003; Bugatti *et al.*, 2014). B cells both respond to and produce the cytokines (including tumour necrosis factor-α, interleukin (IL)-1, IL-6, and IL-17A), and chemokines that promote leukocyte infiltration into the joints, synovial hyperplasia, and angiogenesis. B cells also can act as antigen-presenting cells and can induce T cell activation through the expression of co-stimulatory molecules (Wajid Ali Khan and Ali Khan, 2019). Furthermore, pro-inflammatory cytokines and receptor activator of nuclear factor ligand (RANKL) generated via activated B cells, macrophages, T cells, and synovial fibroblasts increase the differentiation and stimulation of osteoclasts, leading to resorption of bone tissue (Bugatti *et al.*, 2014).

Besides, the contribution of B cells in bone homeostasis is recommended by the recognition that autoantibodies recognising citrullinated vimentin can enhance the differentiation of mononuclear cells to osteoclasts (Bugatti *et al.*, 2014).

Vimentin is a cytoskeleton intermediate filament protein found in cells of mesenchymal origin, including endothelial cells, leukocytes, and smooth muscle cells (Eriksson *et al.*, 2009). It is subjected to citrullination through peptidyl arginine deiminase under high calcium concentrations, which can happen during macrophage apoptosis. Citrullinated vimentin has been determined to have a vital role in the generation of ACPAs (Asaga, Yamada, and Senshu, 1998; Soós *et al.*, 2007). ACPA against citrullinated proteins, such as vimentin, have been found to highly specific markers for RA and other autoimmune conditions (Soós *et al.*, 2007). Besides, B cells can be immunoregulatory via the provision of IL-10 and other mechanisms yet to be explained (Kalampokis, Yoshizaki and Tedder, 2013).

Researches have revealed that the use of an anti-CD20 monoclonal antibody in RA reduces circulating B cells, producing an improvement in illness activity for up to 1 year

(Kneitz, Wilhelm, and Tony, 2004). It is thus evident that B cells perform a pivotal role in the RA pathophysiology and therefore warrant further investigation being a therapeutic target.

T cell-dependent B cell generation of antibodies has been associated with microbial antigen exposure, microbial antigens, and metabolites, such as short-chain fatty acids, actively enhance plasma B cell differentiation in both systemic and mucosal locations (Kim *et al.*, 2016). IgA serves as the primary form of secretory antibody identified at the mucosal surface. It thus plays a significant function in maintaining intestinal homeostasis (Mantis, Rol, and Corthésy, 2011). Possible mechanisms include binding and prevention of uptake of microbial antigens in the lumen, bacterial disruptions and agglutination, neutralisation of pathogenic bacterial toxins, and stimulation of growing bacteria (Zhao and Elson, 2018).

Various mechanisms have been proposed to explain the establishment of mutualism between secretory IgA and intestinal microbiota. Secretory IgA can enhance members of the microbiota, such as *Bacteroides thetaiotaomicron,* to decrease the expression of pro-inflammatory surface epitopes (Peterson *et al.*, 2007). Coating of some luminal bacteria via secretory IgA guides microorganisms entrance into the Peyer's patches, where a germinal centre response is produced, and a positive loop of antigen-specific IgA production is established (Fransen *et al.*, 2015). Microbial antigen identification mediated via various MHC repertoires additionally contributes to modified IgA repertoires, which in turn changes microbiota composition in the intestine (Kubinak *et al.*, 2015).

Due to physical proximity, the intestinal microbiota dramatically affects the production of intestinal IgA (Talham *et al.*, 1999). The reduction of intestinal microbial activation results in fewer numbers of IgA+ plasma cells in the intestine and decreased abundance of IgA (Lécuyer *et al.*, 2014). This is possible because of the compromised development of

isolated lymphoid tissues – a major site for T cell-independent IgA production (Tsuji *et al.*, 2008). Segmented filamentous bacteria potently boosts T-cell-independent IgA production via activation of postnatal development of isolated lymphoid tissues and tertiary lymphoid tissue in the gastrointestinal tract (Talham *et al.*, 1999; Lécuyer *et al.*, 2014). A part of antimicrobial IgA in the intestine is polyreactive and produced from this high-capacity low-affinity pathway (Zhao and Elson, 2018). Nevertheless, the majority of intestinal IgA is T cell-dependent, especially that directed at microbial proteins and is a member of a low-capacity and high-affinity pathway. Here T-dependent IgA mainly happens in the payer's patches through B cells interacting with antigen-loaded dendritic cells into a chemokine Receptor CCR6-Dependent manner (Macpherson and Uhr, 2004; Reboldi *et al.*, 2016).

Microorganisms such as segment filament bacteria and *Mucispirillum sp*. capable of adhering to epithelial cells are potent inducers of T-cell-dependent IgA (Bunker *et al.*, 2015), presumably through activated uptake of their antigens into dendritic cells. IgA-producing B cells home to the intestinal lamina propria, where IgA is generated and then moved across the epithelium into the intestinal lumen through polymeric immunoglobulin receptor expressed on the basolateral side of epithelial cells (Song *et al.*, 1994). Polymeric immunoglobulin receptor deficiency leads to the abrogation of IgM and IgA transcytosis, resulting in increased serum IgG antibodies against intestinal commensals and pathogens, showing the crucial role of secretory antibodies in limiting systemic exposure to microbial antigens (Zhao and Elson, 2018).

Although investigated for several decades, the role of intestinal microbiota in IgA induction was intensely studied in the past few years (Kau *et al.*, 2015; Viladomiu *et al.*, 2017). Further, it has been found that colonisation of germ-free (GF) mice with IgA-coated

bacteria from inflammatory bowel patients exacerbated dextran sulphate sodium-induced colitis (Palm *et al.*, 2014). Moreover, Enrichment of Enterobacteriaceae taxa was distinguished with high IgA coating in Crohn's patients-linked spondyloarthritis and diet-dependent enteropathy, respectively (Kau *et al.*, 2015; Viladomiu *et al.*, 2017).

Intestinal some IgG subclasses and IgM and also associated with gut microbiota, the majority of which are excited through the T-cell-independent pathway (Ehrenstein and Notley, 2010; Koch *et al.*, 2016). Surprisingly, an increasing amount of IgG2b and IgG3 have been found in the secretory compartment in the intestine as well. The production of these antibodies is reliant on Toll-like receptor signalling via B cells (Koch *et al.*, 2016). Moreover, IgM+ plasma cells in the human gut secrete IgM antibodies that assist retain a different community of commensals in the mucus layer in synergy with IgA (Zhao and Elson, 2018). The IgD isotype is rare compared with other antibody isotypes. Still, recently it was shown that IgD class switch recombination happens preferentially in mucosal places and is reliant on a diversified intestinal microbiota (Choi *et al.*, 2017).

1.3.2.3 Natural killer T cells

Natural killer T (NKT) cells are differentiated by an invariant T cell receptor expression, Vα24Jα18 in human beings and the orthologous Vα14Jα18 in murine. NKT cells identify microbial glycolipid components presented by the non-classical MHC member, CD. It has been found that NKT cells and gamma delta T cells are fast responders to antigenic stimulation and are able of generating a variety of immunoregulatory cytokines (Baxter *et al.*, 1997; Akbari *et al.*, 2003; Bendelac, Savage and Teyton, 2007). Inside the gut, NKT cells are defensive in Th1-mediated models of inflammatory bowel disease (Heller *et al.*,

2002). In recent times, it has been demonstrated that microbial stimuli of NKT cells in the mice gut influence NKT cell phenotypic and functional development (Wingender *et al.*, 2012). This indicates that NKT cells perform protective roles in arthritis models (Coppieters *et al.*, 2007) and spondyloarthropathy (Jacques *et al.*, 2010).

The consequences of several surveys (Yanagihara *et al.*, 1999; Aggarwal, Sharma and Bhatnagar, 2013, 2014) were shown that NKT cells and NK cells numbers are remarkably decreased in the peripheral blood of arthritis patients. Obtaining information is proposed that a reduction of NKT cells and NK cells numbers can bring about to the increased illness activity and joint damage as well as bone destruction in rheumatoid arthritis.

1.3.2.4 Gamma delta T cells

There are many cells considered a link between innate and adaptive immunity, for example, gamma delta cells. They are found in massive quantities in intestinal mucosa in comparison with their rate in other areas such as the spleen or lymph nodes (50% to 1,5%) (Wu and Wu, 2012). There are many functions for gamma delta T cells as bearing various antigen receptor particles (TCR) on their cell surfaces and have numerous features of the innate immune system cells, which include Toll-like receptors, the main innate immunity receptors expression, and dectin-1 (Mokuno *et al.*, 2000; Martin *et al.*, 2009). The response of gamma delta T cells to the microbiome is caused by a dectin-1 receptor which identified microbial beta-glucans (Evans *et al.*, 2011). Furthermore, gamma delta T cells secrete a fibroblast growth factor that functions an essential role in changing the intestinal epithelial growth (Boismenu and Havran, 1994).

It is believed that modification of the quantity of gamma delta T cell or the capacity might, subsequently, have a critical impact on the health of the intestine (Wu and Wu, 2012). Even though resident microorganism's loss has a limited impact on the numbers and gamma delta T cells properties, the cytolytic vitality of gamma delta T cells was decreased in GF murine. It is suggested that the microbiome plays an important function in preserving the gamma delta capacity. They are a powerful maker of inflammatory cytokines, for instance, IL-17, TNF-α, and IFN-γ (Lockhart, Green and Flynn, 2006; Shibata *et al.*, 2007). Gamma delta T cells also are capable of producing disease in the collagen produced arthritis model (Roark *et al.*, 2007). It should be recalled that an increase of gamma delta T cells in synovial fluids and peripheral blood of arthritis patients in comparison with healthy control subjects (Gaur, Misra and Aggarwal, 2015). In addition, an elevated level of IL-17 is produced by gamma delta T cells in patients who have ankylosing spondylitis (Kenna *et al.*, 2012).

1.3.2.5 Mucosal-associated invariant T cells (MAIT)

These cells are an innate-like T cells group that are plentiful in a human intestine, blood and liver and excrete a range of proinflammatory cytokines like IL-17 and IFN-γ in reaction to antigenic stimuli (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010; Dusseaux *et al.*, 2011). As NKT cells, MAIT cells carry an invariant T cell receptor (Vα7.2 in human beings) that distinguishes antigen presented via the non-classical MHC as molecule MR1 (Treiner *et al.*, 2003).

MR1-related mucosal-associated invariant T (MAIT) cells differentiate vitamin B metabolites, which are produced by a wide range of microbiota (Howson, Salio and

Cerundolo, 2015). For example, bacterial and fungal communities (Cella *et al.*, 2009; Buonocore *et al.*, 2010). Recent studies have detected that the rate of MAIT cells was decreased in arthritis patients, in comparison with healthy controls, proposing that MAIT cells might play a significant protective role, which prevents from the progression and development of rheumatoid arthritis (Cho *et al.*, 2014).

1.3.2.6 Cytokines

1.3.2.6.1 IL-6

IL6 is one of the most prevalent cytokines presents in both the blood and joints of patients with active RA. It has been shown that in the synovial joints, IL6 is generated via fibroblasts, endothelial cells, synoviocytes, monocytes, and lymphocytes (Hirano *et al.*, 1988; Guerne *et al.*, 1989; Kishimoto, 1992). Higher concentrations of IL6 and IL6 receptor (IL6R) have been observed in the serum of RA patients compared with healthy control subjects, further in synovial fluid than serum, reflecting local generating through the rheumatoid synovium (Hirano *et al.*, 1988; Houssiau *et al.*, 1988; Swaak *et al.*, 1988). Serum IL6 level associated with and radiological joint damage and illness activity. However, another study found the lowering of IL-6 levels through the first twelve months of DMARDs therapy to be the prognostic marker used for clinical outcomes in RA patients (Straub *et al.*, 1997). Synovial fluid with high concentrations of IL 6 activates osteoclast, and the level of activation associates with joint destruction in these patients (Kotake *et al.*, 2009). Though, the specific pathogenic part of IL6 in RA is arguable due to IL6 has both anti-inflammatory and proinflammatory characteristics in vitro. Furthermore, IL-6 has been

associated with illness states such as obesity and diabetes when chronically increased. Forming a relationship between IL-6 and microbial diversity or abundance may establish new avenues for clinical therapy or develop diagnostic evaluations (Cooper *et al.*, 2016). It has been revealed that lactic acid bacteria (such as *Lactococcus,* and *Bifidobacterium*) stimulate the production of interleukin-6 (IL-6) (Miettinen, Vuopio-Varkila and Varkila, 1996). Further, IL-6 is produced via oral bacteria, for example, *S. gorgonii, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, S. sanguinis* and *Inactivated Streptococcus mitis* in both human healthy and atherosclerotic patients (Pessi *et al.*, 2015). Moreover, Proteases excreted through the fungal microbiome, such as *Aspergillus fumigatus* activates the IL-6 production (Borger *et al.*, 1999).

1.3.2.6.2 IL-17

IL-17 is defined as a proinflammatory cytokine generated by human T cells as T helper 17, however, a significant part of the IL-17 released by innate immune cells such as INKT cells and gamma delta T cells through an inflammatory reaction in response to pathogens, stress or injury (Cua and Tato, 2010). IL-17 has been appeared to be generated 4-8 hrs following a bacterial pathogen (Takatori *et al.*, 2008; Kobori *et al.*, 2010). Innate immune cells that produce IL-17 have been observed to colonise mostly in the gut, the skin, and mucosal tissues, and play as the primary line of resistance against pathogens. Furthermore, they enhance neutrophils and cytokines recruitment that are generated by epithelial cells for defensive immunity and, additionally, physiological process regulation, for instance, angiogenesis and tight epithelial junctions (Cua and Tato, 2010). The distinguishing feature of the innate immune reaction is its fast response to the microbial

pathogens by excreting components that mobilise significant quantities of neutrophils by elevating IL-1, IL-6 and tumour necrosis factor (TNF) activation, which enhance tissue penetration, which is critical for dynamic and fast monitoring of microbial infection (Cua and Tato, 2010).

There are some studies demonstrated that raised levels of IL-17 and its receptor is shown in rheumatoid arthritis synovia and tissue cells, and IL-17 can enhance the degradation of murine models joint (Chabaud *et al.*, 1998; Kotake *et al.*, 1999; Ziolkowska *et al.*, 2000; Cai *et al.*, 2001; Honorati *et al.*, 2001).

It shows that there is a link between the physiological characteristics of gut microbiota and stimulation of IL-17A in a physiological manner to the inhibition of pro-inflammatory-based illnesses and also to fight against other microbes. Thus, changes in gut microbiota population can be related to the pathologic expression of IL-17A and then the stimulation of pro-inflammatory-based illnesses (Douzandeh-Mobarrez and Kariminik, 2017). For example, pathologic expression of IL-17A was associated with the increased number of SFB bacteria (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009a). Moreover, gut microbiota population characteristic can be regarded as vital factors for determining the pathological or physiological features of Th17. For example, some proofs approve that adherence bacteria stimulate Th17 variation more efficiently than other microbiomes (Atarashi *et al.*, 2015). The bacteria which has superantigenic, for example, *Staphylococcus aureus,* are identified as critical candidates for the introduction of pathological roles of Th17 in the mucosal immune response (Islander *et al.*, 2010).

1.3.2.6.3 IL-22

It has been noticed that IL-22 plays an essential role in the inflammation, host defence, and tissue homeostasis, and also, the IL-22-IL-22R pathway contributes to regulating inflammation, tissue repair, and immunity (Sonnenberg, Fouser and Artis, 2011). IL-22 is expressed by immune cells existing underneath the epithelium and is induced via microorganisms present in the gut. IL-22 catalyses the epithelial cells through the IL-22RA1–IL-10R2 receptor complex stimulating alterations in the expression of genes included in the preservation of epithelial barrier integrity, with an assortment of functions in pathogens resistance, for instance, tight junction fortification, the secretion of a broad range of bactericidal compounds, and mucus layer changes and hydration. These mechanisms of pathogen resistance, in turn, impact the composition of microbiota communities and generate an environment that eliminates pathogens (Schreiber, Arasteh and Lawley, 2015). Recently, da Rocha et al. have shown that an increased Serum IL-22 in RA Patients with association with disease Activity (da Rocha *et al.*, 2012a).

It has been noted that segmented filamentous bacteria can induce IL-22 expression in the gut of mice (Kolwijck and van de Veerdonk, 2014). Further, *Aspergillus* is a potent inducer of the cytokine IL-22, which induces defensins (proteins identified in human, animals, and plants and their function as antimicrobial peptides which are active against bacteria and fungi) which may affect the pulmonary microbiome composition (Kolwijck and van de Veerdonk, 2014).

1.3.2.6.4 IL-23

IL-23 plays a fundamental role in maintaining the intestinal homeostasis via inducing to secrete antimicrobial proteins as defensins, which limit the connection between microbiota and intestinal epithelial cells (Mease, 2015). IL-23 also is a part of a fascinating collection of cytokines that plays a crucial role in chronic inflammatory illnesses development as rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and psoriasis (Mease, 2015). It is hypothesised that in hereditarily predisposed people, exogenous and endogenous inducement, for example, changes in the commensals, microbial antigens, immunologic modulation, and biomechanical stress, might result in an escalated presence of cytokines as IL-23, which in turn motivate the induction and differentiation of TH17 and other immune cells, which are a part of the innate immune system that elicit adaptive immune activities and inflammatory autoimmune illnesses (Mease, 2015). IL-23 produces IL-17a, IL-17F, IL-6, and TNF-alpha. TH17 activated by the IL-23, which in turn promotes osteoclast development by IL-17 production, which stimulates receptor activator of NF-kappa B ligand and then bone destruction of arthritic patients (H. -R. Kim *et al.*, 2007; Wendling, 2008). Sato and co-works observed that the mRNA expression of RANKL associates with that of IL-23 in synovial tissues of arthritic patients. In RA, IL-23 level associates with IL-17 levels in the synovial fluid as well as with IL-17 and TNF-levels in the serum (Sato *et al.*, 2006). It has been shown that IL-23 activates gut microbiota dysbiosis related to susceptibility to spondyloarthritis and ileitis in ZAP-70 mutant SKG mice (Rehaume *et al.*, 2017).

1.4 Microbiome

1.4.1 General introduction

The term “microbiome” describes the genetic material of all microorganisms (bacteria, fungi, viruses, and protozoa) that reside within and on our bodies. Conversely, the term “microbiota” refers specifically to the viable microorganisms that are found within these populations (Marchesi and Ravel, 2015). Whilst previous estimates state that the number of microbial cells present in our microbiota exceeded our own by a factor of ten, current estimates are more conservative and suggest that the number of bacterial cells in the human body is roughly equal to the number of human cells, representing a mass of approximately 0.2 kg (Sender, Fuchs and Milo, 2016). In either case, the microbiota represents a significant source of non-host biological material. It is further well established that continuous dynamic interactions between the resident microbiota and the human body occur, and that these interactions are required for the maintenance of normal physiology (Choy, 2012). Resident microorganisms obtain from their host a steady source of nutrients, protection, transport, and a stable environment. Simultaneously, they afford various advantages to the host by protecting against infection and colonisation of pathogenic microorganisms, producing substances such as enzymes and vitamin K that support digestion and nutrition, and by modulating the activity and development of the immune system (Choy, 2012).

It should be noted that numerous of these microorganisms are innocuous, as well as fundamental to the host health. However, others can bring about diseases, and are in this way, considered pathogens (Relman and Falkow, 2001). Many factors have been reported

to influence the human microbiota. These include age, diet, sex hormones, taking medications, travel, and another disease (D'Argenio and Salvatore, 2015).

1.4.2 **Microbiome changes in diseases**

Until recently, human microbiology was dependent on the identification of single microbes, for instance, bacteria, fungi, and viruses, often isolated from patients with acute or chronic infections. Novel culture-independent molecular biochemical analyses (genomics, metabolomics, transcriptomics, proteomics) now enable to the identification and classification of diverse microbial populations in a given ecosystem (microbiota), such as the gut and other distal niches, to evaluate all genomes in these ecosystems (microbiome) as well as their gene products (Blum, 2017). These analyses showed that each individual has its microbiota that performs a role in health, such as in immunity, biosynthesis of vitamins or steroid hormones, neurological signalling, as well as the metabolism of drugs. Besides, they significantly provided a new understanding of the role of the microbiome in the pathogenesis of a wide range of human illnesses such as RA. It is predicted that these new insights will translate into diagnostic, therapeutic, and preventive perspectives in the context of personalised/precision medicine (Blum, 2017).

In this section, we critically review and summarise literature reports on the change of microbiome and mechanisms involved in the progress and development of major human diseases, which include ankylosing spondylitis, psoriatic arthritis, obesity, diabetes, atherosclerotic, Inflammatory bowel disease, gout, and cancer.

1.4.2.1 Ankylosing spondylitis, and psoriatic arthritis

Patients with ankylosing spondylitis have been shown to have a distinct gut microbial signature in comparison to healthy people, with a higher abundance of *Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, Rikenellaceae, and Porphyromonadaceae, and lower abundance of Prevotellaceae and Veillonellaceae* (Costello et al., 2015). Further, another study by Zhou and his workers identified that AS-enriched species, including *Prevotella copri, Parabacteroides distasonis, Eubacterium siraeum, Bacteroides coprophilus, and Acidaminococcus fermentans* (Zhou et al., 2020). Pathway analysis showed elevated lipopolysaccharide biosynthesis, oxidative phosphorylation, and glycosaminoglycan degradation in the AS intestinal microbiome. Microbial signatures of AS gut selected by the random forest model exhibited high distinguishing accuracy (Zhou et al., 2020). Some characteristic signatures associated with autoimmunity, such as *Bacteroides fragilis,* were also observed. Lastly, in vitro experiments demonstrated an increased amount of IFN-γ producing cells triggered through a bacterial peptide of AS-enriched species, mimicking type II collagen (Zhou *et al.*, 2020).

These results collectively show that the intestinal microbiome was perturbed in untreated AS patients with diagnostic potential, and some AS-enriched species might be triggers of autoimmunity via molecular mimicry. Additionally, different inflammatory arthritis shared some characteristic microbial signatures.

The intestinal microbiome is also altered in psoriatic arthritis, in which there is overall decreased diversity marked by a lower abundance of *Pseudobutyrivibrio, Coprococcus, Akkermansia, and Ruminococcus*, which correlates with higher concentrations of secretory IgA and lower concentrations of receptor activator of nuclear factor κ-B ligand as well as

decreased concentrations of the medium-chain fatty acids (MCFAs) hexanoate and heptanoate (Scher et al., 2015a). Interestingly, *Akkermansia and Ruminococcus* are both mucin degrading gut bacteria that produce SCFAs and are essential for intestinal homeostasis.

1.4.2.2 Obesity

Obesity is a multifactorial condition resulting in the excessive increase of adipose tissue. The gut microbiome is known to protect gut mucosa permeability and to control the fermentation and absorption of dietary polysaccharides, possibly explaining its significance in the regulation of fat accumulation and the resultant obesity (Muscogiuri et al., 2019).

Recent research into the role of the intestinal microbiome in obesity has revealed some fascinating links. One study investigating this link examined the gut microbiome in obese and lean mice and human subjects (Ley *et al.*, 2005; Ley, 2010). They found that the relative abundance of two dominant types of bacteria, *Firmicutes and Bacteroidetes*, was different in obese and lean subjects. They further investigated the effect of this difference in intestinal microbiota in mice. Changes in the gastrointestinal microbiota that affect metabolism were detected, and the microbiome in obese mice was found to harvest more energy from food than their lean counterparts (Turnbaugh *et al.*, 2006). The authors additionally saw that this effect could be transmitted. Colonising lean mice with microbiota from obese individuals significantly increased total body fat. This study indicates that the gut microbiome plays a vital role in obesity (Ley et al., 2005; Turnbaugh et al., 2006). It reveals that changes in the intestinal microbiome have a significant impact on metabolic function.

The suggested mechanisms by which the intestinal microbiota could contribute to the pathogenesis of obesity include a high abundance of bacteria that ferment carbohydrates, leading to increased rates of short-chain fatty acid biosynthesis, providing an additional source of energy for the host, that is ultimately stored as lipids or glucose (Muscogiuri et al., 2019).

1.4.2.3 Diabetes mellitus

In 2016, the World Health Organization (WHO) expanded the predominance of diabetic disease from current levels to 592 million (12%) in 2035 (WHO, 2016), becoming the third major illness after cancer and cardiovascular disease.

By using genetically engineered diabetic mice (non-obese diabetic, NOD-mice), bacteria in the phylum *Bacteroidetes* (including *S24-7, Prevotella, and unclassified Bacteriodales)* have been demonstrated to protect against the development of type I diabetes potentially. In contrast, increased levels of members of the *Firmicutes phylum*, i.e., *Ruminococcus, Lachnospiraceae, and Oscillospira,* were associated with disease development (Krych et al., 2015). Moreover, through re-deriving NOD-mice as germ-free, the impact of microbiota on diabetes development was assessed (Greiner et al., 2014). Results showed that the absence of microbiota increased inflammation in the islets of Langerhans in the pancreas (insulitis) and decreased glycemic control. At the same time, diabetes incidence was not affected (Greiner et al., 2014). Only a few studies on the gut microbiota have so far been conducted in humans with type I diabetes. In human researchers compared the intestinal microbiome of children aged 1−5years with age-matched healthy controls and identified that children with diabetes had a higher abundance of the combined levels of the phylum

Bacteroidetes and the class *Bacilli* (De Goffau et al., 2014). Healthy children had higher levels of *Clostridium cluster IV* and XIVa (De Goffau et al., 2014). This is in contrast to the NOD mouse experiments and shows important species-differences in host-microbiome interactions.

Regarding bacterial metabolites, healthy children had an increased abundance of butyrate-producing bacteria (De Goffau et al., 2014). Type I diabetic children were also characterised through increased intestinal microbial diversity (De Goffau et al., 2014). This is interesting, as obese and type II diabetic adults generally have lower gut microbial diversity, which may point towards a complex age-related dynamic of the intestinal microbiota development.

The type II diabetic microbiome has lower species diversity and a lower abundance of butyrate-producing bacteria, such as *Faecalibacterium prausnitzii* (Qin et al., 2012; Le Chatelier et al., 2013; Remely et al., 2014). Also, *Clostridium cluster IV and subcluster XIVa* are lower in type II diabetics as compared to lean non-diabetic individuals (Sasaki et al., 2013). Besides, a higher abundance of lactic acid bacteria and *Bifidobacteria* were observed (Sasaki et al., 2013; Remely et al., 2014). Lactic acid bacteria are usually regarded as beneficial, and the relevance of this finding is presently not known. One might speculate whether people with type II diabetes consume a diet with a higher content of sugar, which may promote the growth of carbohydrate-utilising bacteria, such as *bifidobacteria* and *lactobacilli* (Markowiak and Ślizewska, 2017). This illustrates the need for causative studies to delineate which bacterial species in the intestine cause insulin resistance and which bacteria are merely found as a consequence of an unhealthy diet. When performing comparative microbiota studies of healthy versus diseased states in humans, careful documentation of dietary patterns is vital for the interpretation of results.

1.4.2.4 Atherosclerosis

The intestinal microbial composition in healthy people was characterised by increased levels of *Eubacterium* and *Roseburia*, while patients with atherosclerosis had increased abundance of *Collinsella* (Karlsson et al., 2012). Additional studies have also revealed changes in the intestinal microbiota of atherosclerosis patients, as well as the presence of bacterial DNA in atherosclerotic plaques (Koren et al., 2010). Hence, bacteria may influence atherogenesis in several ways, including the reproduction of pro- or anti-atherogenic metabolites, inflammation in plaques, and by modulation of cholesterol metabolism through bile acids (Sayin et al., 2013). Interestingly, the use of probiotic bacteria has shown efficacy in the prevention of atherosclerosis in animal models (Chen et al., 2013), and can decrease biomarkers for cardiovascular risk in humans (Naruszewicz et al., 2002). It remains to be elucidated whether it will be possible to prevent cardiovascular events in humans utilising targeted microbial modulations.

1.4.2.5 Inflammatory bowel disease

It is frequently associated with a state of dysbiosis accompanied through a shift towards an elevated abundance of microbes capable of coping with oxidative stress with a notable increase in facultative anaerobic bacteria of the *Enterobacteriaceae family*. *Ruminococcus gnavus* abundance is also observed to be elevated in inflammatory bowel disease, and certain strains may have evolved to thrive in inflammatory bowel disease intestinal environment by mechanisms of oxidative stress responses (Hall et al., 2017). Antibiotic

usage during pregnancy, but not during infantile age, is positively associated with an elevated risk of developing very early onset inflammatory bowel disease and may be attributed to alterations in the gut microbiome (Örtqvist et al., 2019).

Lactobacillus gasseri SF1138 strain probiotic has been shown to exhibit anti-inflammatory effects in mice models of colitis. It can maintain gut barrier integrity, proposing its protective role against the progression of inflammatory intestinal diseases (Ding *et al.*, 2019). Interestingly, *L. gasseri SF1138* does not modulate the dysbiotic microbiome composition seen in colitis and is suspected of secreting molecules that interact with intestinal cells to protect from inflammation (Di Luccia et al., 2018).

The approach of tungstate-mediated editing of the intestinal microbiome can lessen the severity of intestinal inflammation through inhibiting molybdenum-cofactor-dependent microbial respiratory pathways expressed in specific bacterial populations. They are operational only during episodes of inflammation without significant changes to the microbiome composition (Zhu et al., 2018).

1.4.2.6 Gout

It is a genetic or acquired metabolic disorder with symptoms of severe joint pain with swelling caused by the increase of uric acid synthesis produced from purine metabolic abnormalities (Zhou et al., 2014).

The intestinal microbiomes of patients with gout have been observed to be dysregulated in comparison to healthy individuals, with an elevated abundance of opportunistic pathogens, and similar enrichment was also shown in auto-immune diseases (Shao et al., 2017). *Bacteroides caccase and Bacteroides xylanisolvens* were also found in higher abundance

in gout patients, whereas *F. prausnitzii* and *Bifidobacterium pseudocatenulatum* were seen in lower abundance.

The Microbial Index of Gout, based on the relative abundance of 17 bacterial markers, was suggested as a new method of diagnosis for gout and produced a higher accuracy of 88.9% correlated to conventional blood uric acid tests (Guo et al., 2016).

1.4.2.7 Cancer

Some microbiome taxa, such as *Clostridium* and *Bacteroides*, have been associated with an expansion in tumour growth rate (Jahani-Sherafat *et al.*, 2018). In contrast, other taxa, such as *Bifidobacteria* and *Lactobacillus*, are known to prevent tumour formation (Goulet et al., 2019). As of December 2017, there was preliminary and indirect evidence that the intestinal microbiome might mediate response to programmed cell death protein 1 (PD-1) inhibitors; the mechanism was unknown (Syn et al., 2017).

1.4.3 **Microbial niches**

The resident microbiota is found in different locations in the human body, for example, the gut, oral, and female reproductive system. The distribution of human microbiome locations is revealed in **Figure 1-4**.

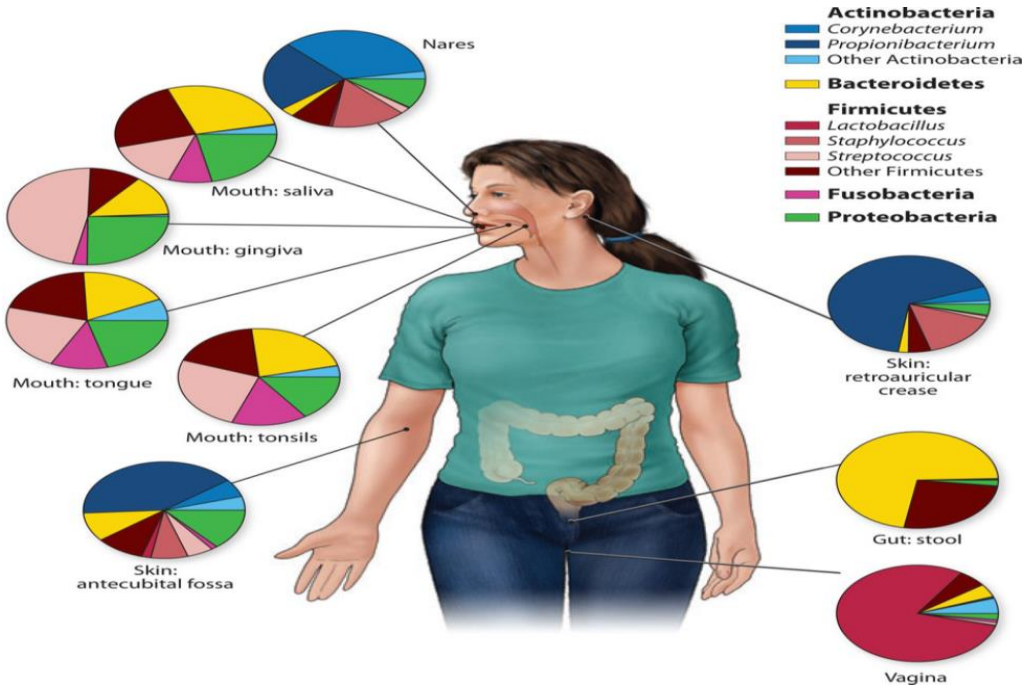


Figure 1-4 "Genus- and phylum-level classification of Bacteria colonising a human body." Each body location is colonised by a particular bacterial taxonomy allocation (Grice and Segre, 2012).

The most abundant microbial community in the human body resides in the intestine. The human gastrointestinal tract contains more than 500 microbial species and roughly 10^{14} bacterial cells (Haller, 2018). Dominant bacterial genera encountered in the various sections of the gastrointestinal tract are observed in **Figure 1-5**.

The anaerobic bacteria, consisting of mainly *Lactobacillus, Bacteroides, Clostridium, Porphyromonas,* and *Bifidobacterium,* are dominant species in the gut microbiota (Bäckhed *et al.*, 2005). However, the microbiome undergoes dynamic changes during the human life span. The alteration of the intestinal microbiome during the human life span is shown in **Figure 1-6.**

The first gut microorganism's colonisation in humans starts during childbirth when the new baby is exposed to microbiota in the vaginal canal of the mother. Various studies propose that infants born through cesarean section are at a higher risk of non-communicable conditions (illnesses that are not transmitted immediately from one individual to another) than infants born by vaginal delivery (Stinson, Payne, and Keelan, 2018).

The large worldwide disparity is found in C-section rates, highest rates being documented in Latin America and the Caribbean region (40.5%), followed by Northern America (32.3%), Oceania (31.1%), Europe (25%), Asia (19.2%) and Africa (7.3%) (Singh, Hashmi and Swain, 2018).

In specific, epidemiological surveys have associated Cesarean birth with raised proportions of autoimmune diseases, obesity, asthma, and allergies (Stinson, Payne and Keelan, 2018). The way of birth has also been correlated with the variation of the infant microbiota, which is bypassed in cesarean births, and this abnormal colonisation of the first-life microbiota is the mediator of later-life negative results noted in infants born by cesarean birth (Dominguez-Bello *et al.*, 2010, Stinson, Payne and Keelan, 2018).

The study by Dominguez-Bello found C-section infants harboured bacterial populations similar to those found on the skin surface, predominated by *Staphylococcus*, *Propionibacterium spp,* and *Corynebacterium*. However, vaginally delivered infants acquired bacterial communities resembling their maternal vaginal microbiota,

predominated by *Prevotella*, *Lactobacillus*, and *Sneathia spp*. These results establish an essential baseline for studies tracking the human microbiome's successional evolution in various body habitats following different delivery modes, and their linked impacts on baby health (Dominguez-Bello *et al.*, 2010). Furthermore, it has been shown that Infants born via cesarean section had lower numbers of *Bacteroides* and *Bifidobacteria* and also found that cesarean deliveries infants were more often colonised by *Clostridium difficile* in comparison to vaginally born infants (Stinson, Payne and Keelan, 2018).

This has led to the increase using the vaginal seeding technique (the transmission of fluid from a mother's vagina containing the vaginal microbiota and swabbing by a cotton gauze or a cotton swab it over the skin, mouth, and nose of cesarean-delivered infants) to encourage the colonisation of a healthy microbiota of infants (Stinson, Payne and Keelan, 2018). Despite a high level of interest in the non-scientific literature, a relative absence of scientific analysis remains. Vaginal seeding has recently been shown to change the microbiota of caesarean born infants to resemble a community more like that of the vaginally delivered (Dominguez-Bello et al., 2016). Nevertheless, although differences appear on the microbial level, there remains a need to show this affects health outcomes, and further high-quality research is needed. There have been some concerns regarding the safety of this practice (Cunnington *et al.*, 2016). The potential transfer of pathogenic organisms (such as *Group B Streptococci*), via vaginal seeding increases the risk of early-onset sepsis, and additional concerns remain regarding the transmission of viruses like HIV and Herpes simplex (Cunnington *et al.*, 2016). However, valid these concerns may be, it is unlikely that it provides a significantly higher risk than if the infant were to be born through vaginal delivery. Until more data is available, it is not very easy to say with any certainty. When assessing infants after delivery, it will be necessary for clear

documentation of vaginal seeding to ensure paediatricians are aware of exposure to vaginal organisms.

From that point, microbiota colonisation contributes to the shaping of the immune system cells of individuals (Milani *et al.*, 2017). Host microbiota evolution may be affected by genetic factors. A non-obese diabetic rodent model shows this. These mice are protected from type 1 diabetes development. It seems that a lack of MYD88 (protein is activated by Toll-like receptor 4 stimulation that drives the expression of the inflammatory gene resulting in proinflammatory cytokine induction of the adaptive immune system) in non-obese diabetic (NOD) mice may bring about increased the *Bacteroidetes* taxon production, which in turn act to activate T regulatory T cells that inhibit inflammatory T cells activation such as T helper17 cells, sequentially, prevent autoimmune diseases formation such as diabetes (Maslowski and Mackay, 2011). Furthermore, the intestinal microbiota in the first years of life is determined by numerous variables such as mother weight, mother breast milk, the way of delivery, and the structure of the maternal microbiome (Isolauri, 2012; Urbaniak, Burton and Reid, 2012).

The cavity of the mouth in human beings is the second-largest microbial reservoir following the intestine. More than 700 microbial species in the oral cavity are identified by molecular biological technologies such as 16S ribosomal RNA techniques (Nibali and Henderson, 2016). 95% of which belong to the phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Spirochaetes* (Nibali and Henderson, 2016). Other phyla consistently detected are *Synergistes* and *Chloroflexi* (Nibali and Henderson, 2016). Predominant microbial communities within different sites of the oral and oropharyngeal region were shown in **Figure 1-7**.

The pulmonary microbiome populations consist of a complex diversity of microorganisms present in the lower respiratory tract, especially on the epithelial surfaces and the mucous layer. These microbiomes compose of microbial communities such as bacteria and fungi. The bacterial population of the microbiota has been more investigated. It contains a core of nine genera: *Prevotella, Pseudomonas, Megasphaera, Sphingomonas, Acinetobacter, Veillonella, Streptococcus, Staphylococcus, Porphyromonas,* and *Fusobacterium* (Hilty *et al.*, 2010; Erb-Downward *et al.*, 2011; Beck, Young and Huffnagle, 2012; Scher *et al.*, 2016).

The nasal microbiome was identified to be predominantly *Actinobacteria, Firmicutes*, and *Proteobacteria,* with *Propionibacteriaceae* and *Corynebacteriaceae* being the most frequent *Actinobacteria* families, while *Staphylococcus aureus* and *S. epidermidis* are the important Firmicutes (Bassis *et al.*, 2014; C. M. Liu *et al.*, 2015). The pulmonary microbiome receives increasing attention regarding its possible role in the development of RA (Scher *et al.*, 2016), but more research is required for definite answers.

The estimation of the number of species existing on skin microbiome has been radically changed by the use of 16S ribosomal RNA to distinguish microbial species found on skin samples direct from their genetic material. Earlier such identifying had depended upon microbiological culture upon which several types of microbiota did not grow and thus were hidden to science (Grice *et al.*, 2009; Byrd, Belkaid and Segre, 2018). *Staphylococcus aureus* and *Staphylococcus epidermidis* were predominant in cultural studies (Elizabeth A. Grice *et al.*, 2008). Nevertheless, 16S ribosomal RNA research identifies that while common, these species make up only 5% of skin microbiota. Though, skin type provides a rich and diverse habitat for microbiota. Several come from three phyla: *Actinobacteria (51.8%), Firmicutes (24.4%), and Proteobacteria (16.5%)* (Elizabeth A. Grice *et al.*, 2008).

In contrast to other human microbiome communities, which typically display a high degree of diversity under healthy steady-state conditions, the vaginal microbiome is generally dominated by one of the *Lactobacillus species L. iners, L. crispatus, L. gasseri, or L. jensenii*. However, more diverse communities with higher numbers of anaerobic bacteria also can be found. It seems to be stable during reproductive age but is strongly influenced by human behaviour (e.g., hygiene, contraception) and ethnic group membership (Ravel *et al.*, 2011). The production of lactic acid by vaginal bacteria causes low vaginal pH. In recent years, attention to the vaginal microbiome was boosted by studies suggesting it a prominent role in shaping the microbiomes of the sterile newborn. Still, improved evidence suggests that differences in the microbiomes correlating with the mode of delivery may be instead caused by the medical conditions leading to the caesarean section (Chu *et al.*, 2017).

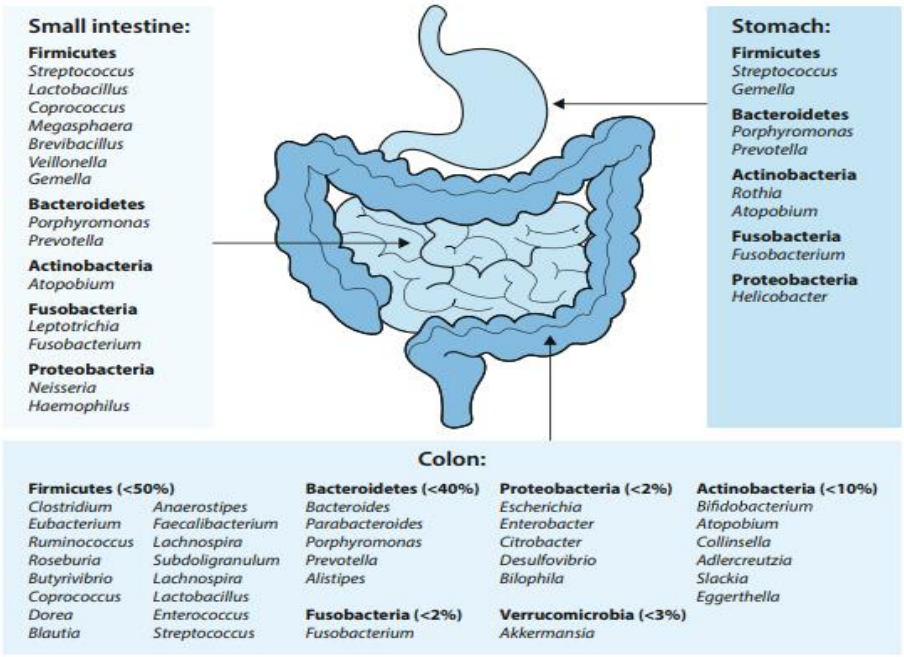


Figure 1-5 Dominant bacterial genera encountered in the various sections of the human gut (Haller, 2018).

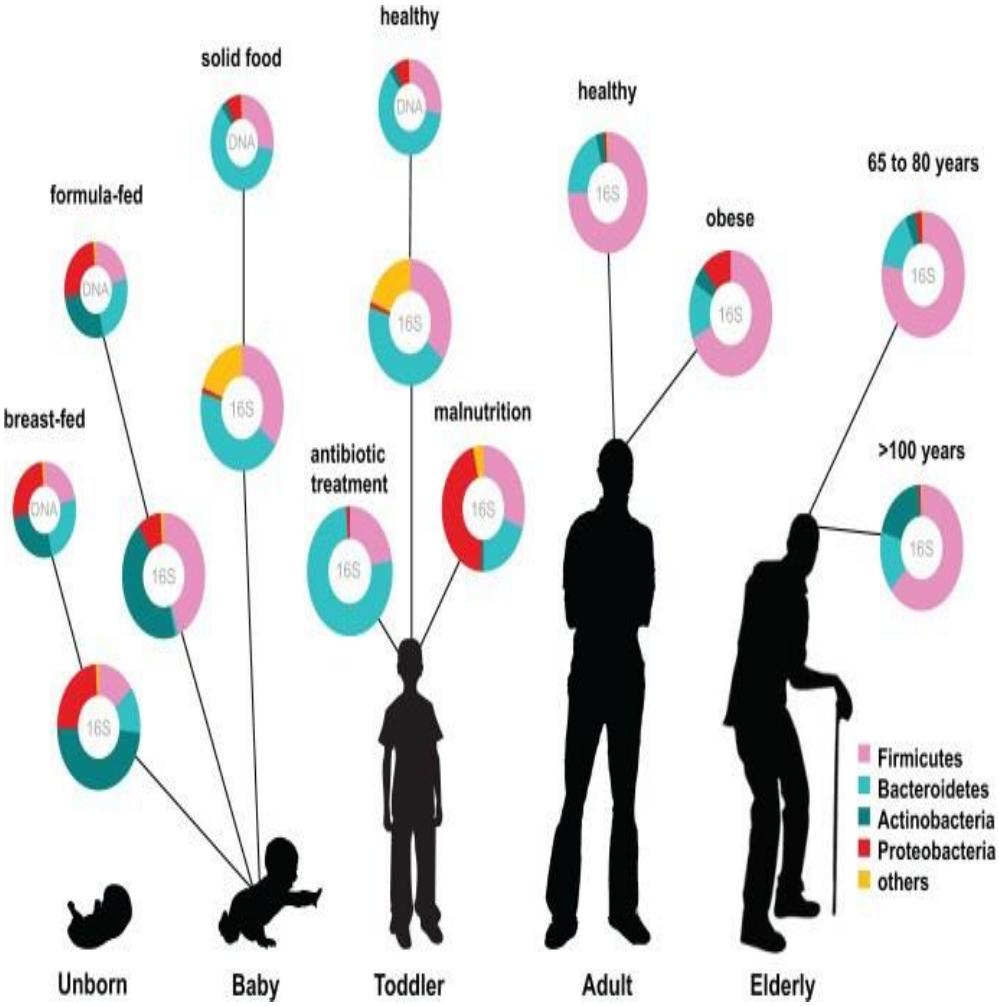


Figure 1-6 changing of the gut microbiome was detected in all human life span. The predominant gut bacteria in new-born and baby are *Actinobacteria* and *Bacteroides* phyla in the toddler. However, the dominant bacterial species in adult and older people is the *Firmicutes* phylum (Hippe et al., 2015).

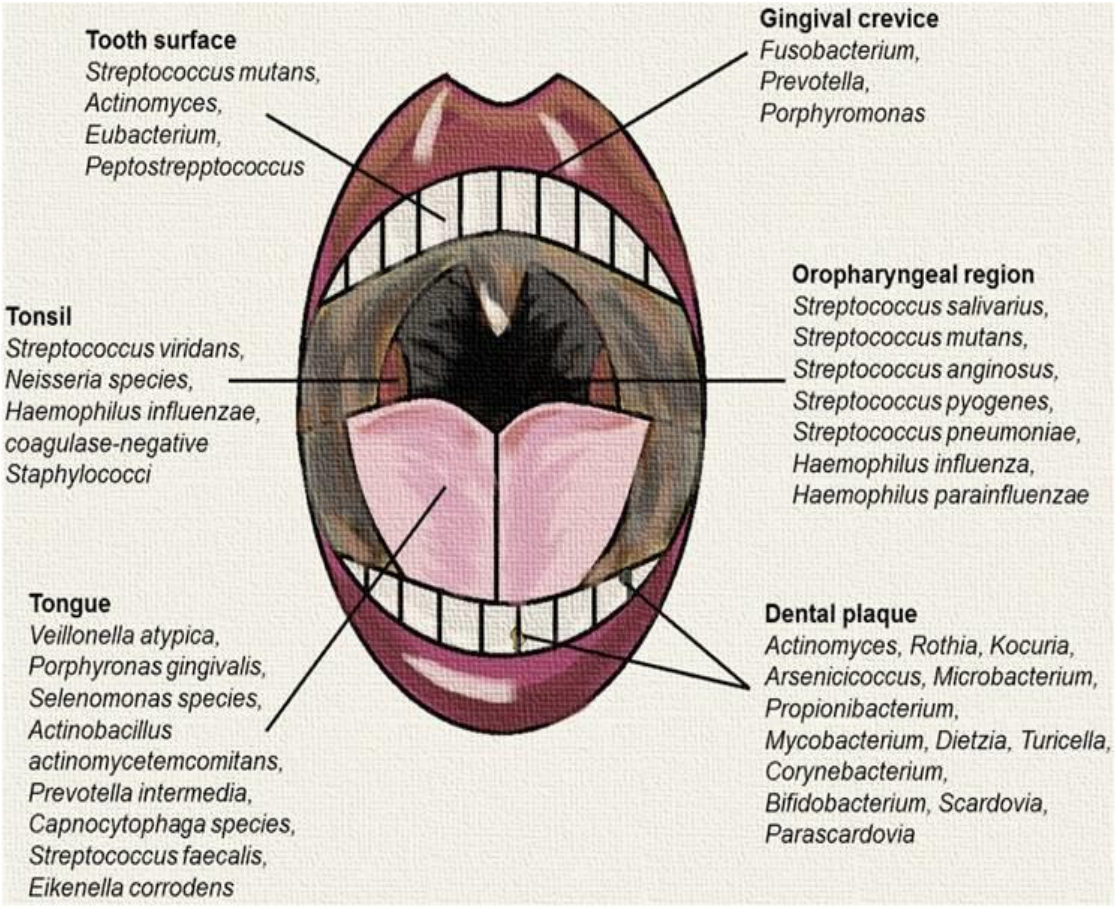


Figure 1-7 Predominant compositions of bacteria colonise the different sites of the oral and oropharyngeal region (Lim *et al.*, 2017).

1.4.4 **Rheumatoid arthritis and dysbiosis**

Several studies have noted that the dysbiosis linked with the human inflammatory disease in different body sites utilising a variety of molecular and biochemical approaches. RA has been associated with dysbiosis of the gastrointestinal tract, oral cavity, lung, urinary tract, and synovial fluid in a variety of studies.

1.4.4.1 Gut microbiome and RA

The significance of the gut microbiome in arthritic disorders was identified when specific microbes were associated with the evolution of reactive arthritis. Findings demonstrated that the synovial tissue of arthritis patients contains a specific microbial DNA such as *E coli, Staphylococcus epidermidis, and Propionibacterium acnes* (Karen E. Kempsell et al., 2000; Taneja, 2014).

The importance of a meta-taxonomic approach is to characterise distant microbial niches through the analysis of microbial nucleic acids and to detect dysbiosis in these niches, which may affect the immune system, which could be a biomarker for RA diagnosis and treatment. However, it is necessary to point out to perform culture experiments, especially at the time when the laboratory is running aerobic and anaerobic cultures for other studies to detect viable colonies of living microbes, which may affect the immune system.

It is worth mentioning that the changed numbers of the intestinal microbiome can influence the whole immune system in the intestine with the following modification in the systemic immune system (Eerola *et al.*, 1994). In the primary finding to demonstrate the relationship

between the microbiome and RA, the fatty acids of gastrointestinal bacteria were analysed to show that arthritic patients with erosive illness differed from healthy individuals (Eerola *et al.*, 1994). In the following study, faecal materials from arthritis patients were examined by 16S ribosomal RNA sequencing of bacteria utilising the reverse transcription-polymerase chain reaction (rt PCR). This result showed that patients who suffer rheumatoid arthritis have a low abundance of *Bacteroides* and *Bifidobacterium* bacterial genera in comparison with healthy controls, proposing dysbiosis (an imbalance of bacteria in the gut) as these are the predominant genera in individuals (Vaahtovuo *et al.*, 2008). On the other hand, the data were based on only a small population, and further confirmation is required. However, the faecal microbiome might not reflect the whole microbial of the intestine.

Scher and co-workers (Scher *et al.*, 2013) tested stool specimens of new-onset arthritis patients and healthy individuals utilising the sequence 16S ribosomal RNA of faecal microbiota. The results found that the *Prevotella copri* abundance with *Bacteroides* absence in new-onset arthritis patients was different, proposing *P. copri* may be pathogenic. Pathogenicity of *P. Copri* was explained by the immune response generated by the DR-presentation of a 27-kD protein from *P. copri,* which could stimulate T-helper-cell 1 (Th1) responses in untreated RA patients (Taneja, 2014). The interesting point of this study is that the proportional abundance of *P. Corpi* associated with the human leukocyte antigen (HLA) alleles presence that shares the sequence of the third Hypervariable region is known “rheumatoid epitope” proposing a change in the gut microbiome before the clinical phenotype appearance. The inflammation in rheumatoid arthritis starts considerably before the actual beginning of the disease (Taneja, 2014). These investigations, combined with humanised rodent, propose that the Major Histocompatibility Complex (MHC) genes (a group of genes that code for proteins presents on the surfaces of cells that assist the immune system in recognising foreign substances, MHC proteins are identified in all higher

vertebrates, In humans, the complex is also known as the human leukocyte antigen (HLA)), play a fundamental part in the bacterial flora settlement in the intestine and consequently indirectly determining the pro-inflammatory conditions in the intestine (Taneja, 2014). Additionally, the probability that RA patients are taking drugs that can regulate their gut microbiome before being observed in a clinic is high (Taneja, 2014).

Zhang and his colleagues (Zhang *et al.*, 2015) showed that the dysbiosis of the gut microbiome of arthritic patients; however, it was partially resolved after RA therapy with methotrexate (MTX). The patients who showed improvement after MTX treatment had an abundance of microbial linkage groups (MLGs) similar to healthy control samples. *Prevotella maculosa* revealed an increased abundance in the healthy control group, as well as in patients who noted improvement following treatment. Furthermore, after MTX therapy, in the gut microbiome, MLGs, including *Veillonella* was abundant in RA patients but showed a decrease after MTX therapy, proposing that MTX therapy could change the intestinal microbiome patients with RA. This proposes that MTX treatment has the potential to turn a 'diseased' microbiome toward a 'healthy' microbiome. (Zhang *et al.*, 2015). Recently, Jubair et al. noted that gut dysbiosis triggers collagen-induced arthritis by mucosal immune responses. Dysbiosis and mucosal inflammation lead to the development of the CIA (Jubair *et al.*, 2018). Therapy with antibiotics such as ampicillin, neomycin, vancomycin, and metronidazole was found to decrease the illness severity, as well as serum inflammatory cytokine and the levels of anti-type II collagen antibodies. Accordingly, specific gut commensal microbiota is sufficient to produce arthritis in rats (Jubair *et al.*, 2018).

A North American survey of chronic RA patients indicated RA patients to be low in stool *Faecalibacterium* and increase rare bacteria such as *Eggerthella* and *Collinsella* (Chen *et al.*, 2016).

The study via Wu et al. (Wu *et al.*, 2017) found that the gut of RA patients was associated with significantly increased levels of *Prevotella copri, Bacteroides, Paraprevotella, Porphyromonadaceae,* and *Phascolarctobacterium* and significantly decreased levels of *Faecalibacterium, Roseburia, Subdoligranulum, Ruminococcus, Pseudobutyrivibrio,* and *Carnobacteriumgroup* when compared those with controls.

Recently, it has been shown that the composition and diversity of the microbiome of patients with RA differed from those in healthy control people in China. An increase in *Escherichia-Shigella* and *Bacteroides* combined with decreases in *Lactobacillus, Odoribacter, Alloprevotella, and Enterobacter* appear to be characteristic of RA in patients from Shanghai, China (Sun *et al.*, 2019). Besides, spearman correlation analysis of physiological blood measures of RA identified those bacterial genera such as *Ruminococcus* and *Dorea* were positively associated with RF-IgA and anti-CCP antibodies (Sun *et al.*, 2019). Further, *Prevotella-2* and *Alloprevotella* were positively associated with C-reactive protein and, *Alloprevotella* and *Parabacteroides* were positively associated with the erythrocyte sedimentation rate, both biomarkers of inflammation. These results suggest that the gut microbiota may participate in RA development through interactions with the host immune system (Sun *et al.*, 2019).

Furthermore, elevated levels of antibodies directed towards specific intestinal microbiome antigens in rheumatoid arthritis patients propose a pathogenic relationship among these microbes and rheumatoid arthritis (Scher and Abramson, 2011).

It has suggested that inflammation of the joints may be caused by the exposure of genetically susceptible individuals, who have the human leukocyte antigen - B27, to degraded products of the gut bacteria. It is suggested that this exposure occurs locally in the joints (Taneja, 2014).

Bacterial ingredients from *Salmonella, Shigella, Yersinia, and Campylobacter* are well-characterised triggers for HLA-B27- associated reactive arthritis (Lin *et al.*, 2014). Cross-reactivity between monoclonal antibodies to HLA B27 and specific Gram-negative bacteria has been published (Rosenbaum and Davey, 2011). There is also sequence homology between a nitrogenase from some bacteria such as *Klebsiella* and HLA-B27, although the importance of these results has been debated (Yang *et al.*, 2016). Raising HLA-B27 transgenic rodents in a germ-free environment limited both intestine and joint inflammation that otherwise spontaneously happens in these rodents (Van De Wiele *et al.*, 2016). The subsequent studies revealed that mono-association of HLA-B27 transgenic mice with *Bacteroides vulgatus* in a germ-free environment was sufficient to re-establish colitis in these rodents (Van De Wiele *et al.*, 2016). A number of studies have shown marked effects of HLA-B27 on innate immunity and host defence in vitro, including effects on the bacterial invasion of cells, intracellular signalling, intracellular persistence, and cytokine production (Penttinen *et al.*, 2004; Sahlberg *et al.*, 2007; Ruuska *et al.*, 2013). Recent evidence proposes that innate immune expansion (dendritic cells or macrophages) and Th17 activation via HLA -B27 molecules may precede the development of microbiome dysbiosis and intestinal inflammation in HLA -B27 transgenic mouse model (Gill et al., 2018).

Penttinen et al. (Penttinen *et al.*, 2004) noted that HLA-B27- transfected monocytes had enhanced inflammatory responses to lipopolysaccharide located on bacterial cell walls. Furthermore, disease-prone HLA-B27 transgenic rats show a variety of dendritic cell abnormalities (Fert *et al.*, 2008; Dhaenens *et al.*, 2009), including disrupted trafficking of dendritic cells from the intestinal tracts to the mesenteric lymph nodes (Utriainen *et al.*, 2012). It is likely that a dysregulated immune response in HLA-B27 individuals alters the

composition of the intestinal microbiome, and that in turn, the modified gut microbiota participates in disease pathogenesis.

It is worth mentioning that the presence of microbial cell wall products which are present in arthritic joints and the changed numbers of intestinal bacteria can influence the immune system in the intestine with the following modification in the systemic immune system (Eerola et al., 1994; Grard *et al.*, 2001; Vaahtovuo *et al.*, 2008).

The nature of the gut microbiota determines the reservoir of lipopolysaccharide (LPS), which is capable of migrating from the intestine into the circulation, where it participates in the inflammation. It is acknowledged that lipopolysaccharides are the main endotoxin components of Gram-negative bacterial cell walls (Jubair *et al.*, 2018).

There is evidence for significant involvement of lipopolysaccharide shed by dormant and resuscitating bacteria as underpinning the chronic inflammation characteristic of a variety of conditions and proposed that LPS may play a role in the RA pathogenesis (Kell and Pretorius, 2015).

It is known that lipopolysaccharide stimulates the CIA. The systemic injection of LPS from S. typhimurium, S. enteritidis, and K. pneumoniae resulted in the stimulation of CIA in mice that was associated with the increased production of IgG2a antibodies and anti-CII IgG as well as the enhanced secretion of cytokines including IL-1β, IL-12, IFN-γ, and TNF-α. Therefore, LPS may play a role in the exacerbation of RA disease (Yoshino and Ohsawa, 2000). It also has found by another study by Tanaka et al. to have marked increases in the degree of expression of mRNA of inflammatory mediators such as interleukin (IL) -1β, tumour necrosis factor-alpha (TNF-α), and macrophage inflammatory protein-2 (MIP-2) in their arthritis paws and of serum anti-CII antibody concentration before the start of arthritic conditions induced through LPS injection (Tanaka *et al.*, 2013). The gene expression was

quick and continuous after the direct activation of nuclear factor κB. The amounts of mRNA of TNF-α, IL-1β, and MIP-2, as well as of matrix metalloproteinases and the receptor activator of nuclear factor κB ligand, increased with the development of arthritis, associated positively with clinical severity and corresponded with histopathological changes (Tanaka *et al.*, 2013). Furthermore, anti-TNF-α neutralising antibody inhibited the development of LPS-accelerated CIA, and a single injection of recombinant mouse TNF-α induced increases in anti-CII antibody concentrations, suggesting that TNF-α may contribute to the development of arthritis through both initiations of inflammation and production of autoantibodies (Tanaka *et al.*, 2013). This indicates that the exacerbation of RA via LPS is associated with the rapid and continuous production of inflammatory mediators and autoantibodies.

A recent study reported that the systemic diffusion of bacterial lipopolysaccharide positively associated with joint inflammatory response and the severity of joint degradation (Drago *et al.*, 2019). LPS can also be concentrated into the synovial fluids and upregulate specific pro-inflammatory cytokines. These immunological factors can have a vital role in the pathogenesis of arthritis, particularly in RA (Drago *et al.*, 2019).

Other components derived from pathogens can cause or potentiate arthritis in animal models. For instance, peptidoglycan extracts from the bacterial cell wall of *Streptococcus, Lactobacillus Bifidobacterium,* and *Collinsella* can induce chronic arthritis in certain susceptible rat strains (Yinshi Yue, 2013). Peptidoglycan, a bacterial cell wall component, is a potent arthritogenic. It can stimulate lymphocytes and induce the production of cytokines and polyclonal autoantibodies, including RF in vivo using animal models and

vitro utilising cell culture systems (Yinshi Yue, 2013). Further, bacterial lipopolysaccharide potentiates type II collagen-induced arthritis in mice (Yinshi Yue, 2013).

It has been established that intestinal bacterial cell wall offcasts are associated with RA. Intestinal microorganisms continuously shed peptidoglycans from their cell walls as they grow and divide, and these offcast molecules can cross the gut barrier and enter the circulatory system (Huang *et al.*, 2019). Huang and co-workers developed an anti-muramyl-l-alanine-isoglutamine (MDP) antibody termed 2E7. This antibody enabled them to quantify and neutralise serum peptidoglycan via targeting MDP, a conserved structure within peptidoglycan. Utilising 2E7, the researchers proved the presence of MDP in the blood of all but 4 of the 340 healthy people tested, albeit at varying concentrations (Huang *et al.*, 2019).

Interestingly, serum concentrations of MDP were higher in patients with rheumatoid arthritis (n=84) than in healthy people, proposing a link to the RA system (Huang *et al.*, 2019). Increasing the concentration of MDP in mice utilising an osmotic pump exacerbated collagen-induced arthritis (CIA) matched with the healthy subject, whereas administering 2E7 to decrease the concentration of MDP in mice lessened CIA severity compared with giving a control antibody (Huang *et al.*, 2019). In the Collagen Antibody-Induced Arthritis (CAIA) model, administration of 2E7 before the initiation of disease almost completely inhibited the development of arthritis. Earlier studies have determined that MDP is recognised by the pattern recognition receptor NOD2, which triggers proinflammatory signalling pathways. Accordingly, 2E7 was unable to prevent CAIA in mice that lacked NOD2, suggesting that neutralisation of MDP by 2E7 ameliorated arthritis by reducing NOD2 signalling (Huang *et al.*, 2019).

The gut microorganisms can bind toll-like receptors (TLRs) and nod-like receptors to stimulate the immune system, as well as produce metabolites called short-chain fatty acids

that can directly interact with the host (Mcdonald and Nunez, 2005; Hasegawa *et al.*, 2006; Reichardt *et al.*, 2014). Well-Balanced microorganisms maintain immune responses through interaction with intestinal epithelial cells, which helps to maintain a tolerant state within the gut. To date, research has confirmed that the gut microbiome has a profound connection with the host immune system and illness (Belkaid and Hand, 2014). In genetically predisposed individuals, antibiotic exposure, environmental factors like inappropriate diet can cause dysbiosis in the gut microbiota resulting in the expansion of contraction of certain species of a genus (Taneja, 2014; Mikkelsen, Allin and Knop, 2016). An alteration to a single bacterial taxon and/or the whole community leads to an imbalance between the pathobiome and the symbiome immune responses with a breakdown of self-immune tolerance, prompting several autoimmune diseases such as RA (Mohammed and Elmakhzangy, 2017). For example, mono-colonisation of a germ-free mouse with a single gut-microbiome "segmented filamentous bacterium is sufficient to produce fully functional TH17 cells that generate proinflammatory cytokines IL-17 and drive the onset of arthritis. The segmented filamentous bacterium upregulates the producing of acute-phase isoforms of serum amyloid A in the ileum, which can act on dendritic cells from the small intestinal lamina propria to provoke Th17 differentiation (Ivanov *et al.*, 2009b; Wu *et al.*, 2010). Moreover, mono-colonisation with *Lactobacillus bifidus* in IL-1 receptor antagonist knockout mice resulted in the rapid onset of arthritis, which was dependent on Toll-like receptor activation via *L. bifidus*. The arthritis onset was preventable through promoting a germ-free environment (Abdollahi-Roodsaz *et al.*, 2008). Besides, colonisation of the mice intestine with *Prevotella copri* can enhance experimental dextran sulphate sodium-induced colitis, which can initiate a proinflammatory drive in human arthritis. The *Prevotella copri* genome encodes phosphoadenosine phosphosulfate reductase, an oxidoreductase that contributes to the production of thioredoxin. Thioredoxin is a cellular reducing catalyst

induced via oxidative stress and is involved in the redox regulation of transcription factors such as NF-kappa B. Thioredoxin has been widely involved in the pathogenesis of RA with significantly increased concentrations observed in both serum and synovial fluid of RA (Yoshida *et al.*, 1999; Mohammed and Elmakhzangy, 2017). Further, commensal microorganisms can secrete large amounts of adenosine 51-triphosphate that can stimulate a unique subset of lamina propria cells, CD70highCD11clow cells. The $D70^{high}$ $CD11c^{low}$ subset cells could express Th17-inducing molecules, for instances, transforming-growth-factor, IL-6, and IL-23, and produce Th17 differentiation (Mohammed and Elmakhzangy, 2017).

The classical assertion that RA lacks a microbial component stems from the generalised inability of microbiologists to see microbial colonies on artificial media when synovium samples from RA patients are subjected to *microbiological culture.* Such assessments crudely characterise organisms as “alive” or “dead” (or possibly more accurately, present or not present) based upon this colony-forming ability, yet do not routinely identify the potential for the existence of viable-non culturable bacteria (Hammad, Liyanapathirana and Tonge, 2019).

Moreover, extensive evidence now provides for the presence of free bacterial DNA or RNA, and viable-non culturable bacteria in affected joints, leaving the possibility of;

1) Microbial DNA found within the bloodstream translocates from the gut, a process termed atopobiosis, followed by killing these microorganisms by an immune cell known as a phagocyte leaving only the DNA following the passage of cell-free microbial nucleic acid to the synovium via the circulatory system (as evidenced by the simultaneous presence in both fluids).

2) Whole viable bacterial translocation is followed by a state of dormancy due to activation of the immune system or to unfavourable environmental conditions (Hammad, Liyanapathirana and Tonge, 2019).

With relevance to the primary approach, it is well confirmed that the immune system could distinguish self from non-self-nucleic acid (DNA and RNA) in the human blood and synovial fluid via specific pattern recognition receptions (Chi and Flavell, 2008) and that TLR9 recognises foreign DNA, and RNA is identified via TLR3 on immune cells, producing in the upregulation of various pro-inflammatory cytokines such as TNF *alpha, and IL-6,* which are associated with *RA* (Atianand and Fitzgerald, 2013; Castañeda-Delgado *et al.*, 2017). With importance to the latter scenario, dormant microorganism's cells or viable-non culturable bacteria may colonise the joints of RA patients, unidentified by routine culture, whilst retaining the ability to shed inflammatory agents such as lipopolysaccharide (LPS) and other antigenic components (Pretorius *et al.*, 2017). It showed that bacterial LPS could activate many of the common RA-associated cytokines, for instance, TNFα, the IL-1 family, IL-6, IL-12 family, and IL-15 (Rossol *et al.*, 2011; Li *et al.*, 2014).

While detailed studies of the gut and other niches dysbiosis have been performed in RA patients, however, previous studies of these sites have yielded interesting perturbations that appear to associate with disease presence. Studies of the other human microbiomes are absent (significantly including the blood), and to date, no study has attempted to link microbiome changes across discrete sites. The novel aspect of the blood work is to find microbiome signatures in the blood.

Wiest and co-workers suggested that the gut microbiome may enter the blood from the gut by three approaches (See Figure 1-8). Firstly, via dendritic cells, which are not reliant on the reduced tight junction (An intercellular barrier between epithelial cells within the gut that

is vital to the role of the physical intestinal barrier, regulating the paracellular passage of various substances including water, ions, solutes across the intestinal epithelium). Secondly, microorganisms may enter the blood through the inflamed or damaged epithelium with a defective epithelial wall. Finally, by microfold cells (M-cells) which lie on top of Peyer's patches and function as specialised cells providing entrance of microbial components to antigen-presenting cells (Wiest, Lawson and Geuking, 2014)

As microbiome dysbiosis in distal niches such as the gut has been well represented in RA disease, and the blood microbiome is prophesied to be derived from microbial translocation from other body habitats, it is highly expected that the development of RA would have a significant influence on the blood microbiome composition given that microbial dysbiosis is a well-described hallmark of RA disease. No publications on RA disease and alterations in the blood microbiome have been published to date, barring the published work resulting from this thesis (Hammad *et al.*, 2020).

The association of RA state with alterations in the blood microbiome are likely to reflect microbial dysbiosis at distant body sites. Characterisation of the blood microbiome, therefore, offers potential opportunities for novel biomarker and therapeutic developments.

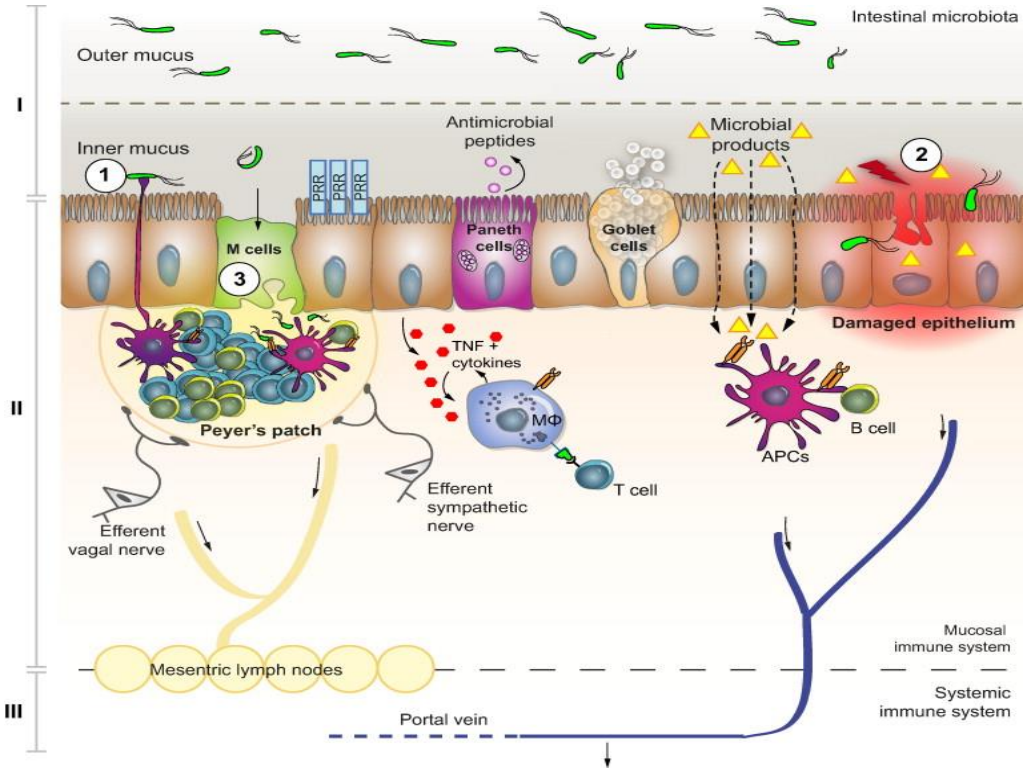


Figure 1-8 Members of the gut microbiome may enter the blood from their place of habitation. By three approaches; firstly, via dendritic cells, which are not reliant on the reduced tight junction. Secondly, the microbiome may enter the blood through the inflamed or damaged epithelium with a defective epithelial wall, finally, by microfold cell (M-cells), which lie on top of Peyer's patches and function as specialised cells providing entrance of microbial components to antigen-presenting cells. Furthermore, three different levels of barriers (I–III) against bacterial translocation are observed: (I) lumen and secretory component (e.g., inner and outer mucus layer, antimicrobial peptides) of the intestinal barrier; (II) mechanical epithelial barrier and the gut-associated lymphatic tissue beneath with response elements to bacterial translocation (e.g., TNF and other pro-inflammatory cytokines) and autonomic nervous system; (III) systemic immune system as the third barrier in case of spreading of bacteria (I products) beyond the mesenteric lymph nodes including hematogenous (portal venous) and lymphatic (ductus thoracicus) way of delivery. TNF, tumour necrosis factor; APC, antigen-presenting cell; PRR, pattern recognition receptors (Wiest, Lawson and Geuking, 2014).

1.4.4.2 Oral microbiome and RA

According to several studies, the commensal bacteria are implicated in the aetiology of periodontitis (gum illnesses) (Löe, Theilade, and Jensen, 1965) and tooth decay (van Houte, 1994). Furthermore, they may also play a significant part in the pathogenesis of some autoimmune illnesses, for example, RA (Zhang *et al.*, 2015), diabetes (Genco *et al.*, 2005), and arteriosclerotic vascular disease (Scannapieco, Bush, and Paju, 2003). It is interesting to note that the rate of tooth loss and inflammation of the tissue around the teeth of arthritis patients are higher than healthy people (de Pablo, Dietrich, and McAlindon, 2008). Moreover, the severity of gum disease has been positively associated with RA disease activity (Choi *et al.*, 2016). It should be mentioned that human leukocyte antigen (HLA) genes are associated explicitly with arthritis disease and periodontal disease as HLA-DRB1 locus. The primary HLA marker for both illnesses is the HLA-DRB1 locus (gene belongs to the MHC class II gene, which supplies instructions for generating a protein that existent on the surface of specific immune cells) (Zhang *et al.*, 2015).

There are some findings to indicate that the treatment of people who have periodontal illnesses seems to bring about the decreased activity of the disease in arthritis patients (Ortiz *et al.*, 2009). Periodontal diseases are prevalent oral inflammatory illnesses that occur in response to bacterial plaque biofilms such *as Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis,* which cause damage to the periodontal ligament, the gingiva (gums), and alveolar bone (Cheng, Meade and Devine, 2017). RA and Periodontal diseases detect some pathogenic similarities, for instance, the immune response of the host which leads to inflammation of soft tissue with after that, hard tissue damage, and specific risk factors, involving obesity and smoking, though several

studies only reveal associations at specific stages of illness aetiology (Cheng, Meade and Devine, 2017).

Recently, DNA of oral bacteria, for instance, *Prevotella intermedia (P. intermedia), Porphyromonas gingivalis (P. gingivalis), and Tannerella forsythia* (*T. forsythia*) with other oral pathogens, such as *Prevotella nigrescens*, and *Treponema denticola* and *Fusobacterium nucleatum* have been identified in the synovial fluid of arthritis patients and some spondyloarthritis patients. Moreover, increased levels of antibodies directed against some periodontal bacteria such as *T. forsythia, P. intermedia,* and *P. gingivalis* have been revealed in both the serum and synovial fluid of arthritis patients (Karen E. Kempsell et al., 2000; Moen et al., 2005; Loyola-Rodriguez et al., 2010; Témoin et al., 2012a).

The oral microbiome can enhance immunological reactions via several mechanisms. One of these mechanisms is used by *P. gingivalis* that is a gram-negative bacterium that is found in the gingival clefts of people who bear inflammatory response in the gum as well as in healthy people. This microorganism has the capacity to release peptidyl arginine deiminase (PAD) that brings about the citrullination (the alteration of the amino acid arginine in a protein into the amino acid citrulline that can excite the immune system to release autoantibodies which take part in the pathogenesis of autoimmune illness such as RA) (du Teil Espina *et al.*, 2019). Zhang et al. analysed salivary and dental samples of arthritic patients that identified a dysbiotic oral microbiome relative to healthy subjects. They also have been observed *that Haemophilus species*, which were low in the oral of RA patients, and which negatively linked with autoantibodies compared to RA. In contrast, the *Lactobacillus salivarus* was increased in both salivary and dental samples of arthritis patients and positively associated with illness activity (Zhang *et al.*, 2015).

The study by Lopez-Oliva et al. also characterised the oral microbiome in periodontally healthy people with or without rheumatoid arthritis (Lopez-Oliva *et al.*, 2018). The study showed that the proportion of *Prevotella* is prominent in the oral microbiome of RA patients than healthy populations and, similarly to a study by Scher et al., *Leptotrichia spp*., proposing a possible significant role for these two pathogens in the induction of RA (Scher *et al.*, 2012; Lopez-Oliva *et al.*, 2018). Further, *Cryptobacterium curtum* was found as the dominant species in arthritis patients (Lopez-Oliva *et al.*, 2018). This is of importance notably due to *C. curtum* ability of citrullinating free arginine via the arginine deiminase pathway. Still, Antibodies to Citrullinated Protein Antigens (ACPAs) target citrullinated proteins, and not free citrulline. It has additionally been indicated that the synovial fluid of arthritis patients has antibodies directed against heat shock proteins of *P. Intermediate and Prevotella nigrescens,* and these antibodies might cause an immune response in RA condition (Yoshida *et al.*, 2001; Témoin *et al.*, 2012a). The oral microbiome can invade the blood circulation as a consequence of bacteremia (microbiome presence in the bloodstream), and this frequently occurs the following chewing or after tooth brushing and dental procedures such as periodontal probing or ultrasonic scaling (Kinane *et al.*, 2005; Forner *et al.*, 2006). It is believed that the oral microbiota colonises an assortment of tooth surfaces, including gingival cleft fissures and smooth surfaces resulting in dental plaque formation (Loyola-Rodriguez *et al.*, 2010). On contact with this tissue-based immune system, it causes tissue cell mobilisation and producing IL-1, IL-6, and TNF-a, following tissue damage by activating matrix metalloproteinases (MMPs) production (a group of enzymes which act to degrade helical fibrillar collagen found in teeth, cartilage, and bone) (Loyola-Rodriguez *et al.*, 2010). Therefore, it is plausible that the oral microbiome might reach distant locations of the human body and be implicated in the pathogenesis of diseases such as autoimmune diseases.

1.4.4.3 Blood microbiome and RA

Many normal resident microbiotas are opportunistic pathogens and have the potential to induce disease if there is a break in resistance of the host. The breach of resistance barriers enables microbiota to overpopulate their usual habitat and also penetrate areas of the body that they are not usually located (Burd and Westblade, 2017). Recent studies have further revealed that evidence of the microbiome (generally at the nucleic acid level) is detectable in the circulatory system, and purports that this is the result of microorganisms (or parts thereof) moving from their usual place of habitation such as the gut, oral cavity, respiratory tract into the blood; a process termed atopobiosis (Potgieter et al.,2015).

It is almost universally accepted that the circulatory system of healthy humans is sterile based upon our general inability to detect proliferating microorganisms (Potgieter *et al.*, 2015). However, there are exceptions to this belief, with a number of studies reporting the presence of proliferating organisms in the circulation of apparently healthy subjects (Granfors *et al.*, 1998; Nikkari *et al.*, 2001; Damgaard *et al.*, 2015; Païssé *et al.*, 2016; Gosiewski *et al.*, 2017). The identification of microorganisms or their components and or products in human blood, such as lipopolysaccharide utilising culture or immunological assays, is evidence of the existence of these microorganisms in blood, albeit scarce (Burd and Westblade, 2017). However, culture-positive bacteremia following tooth brushing and oral irrigation devices is well appreciated (Berger *et al.*, 1974; Maharaj, Coovadia, and Vayej, 2012). Suggesting, the transient presence of organisms within the circulation is well-tolerated in the healthy host. Besides, numerous investigations report the detection of bacterial 16s rRNA sequences in human blood samples in health and various diseases (diabetes, obesity, cardiovascular disease, atherosclerosis and in Kawasaki disease)

(Amar *et al.*, 2011, 2013; Vajro, Paolella and Fasano, 2013; Trøseid *et al.*, 2014; Abe *et al.*, 2015). These studies pose various questions around the nature of their existence and their function in health and illness and also support the notion that bacteria can circulate in the blood circulation of well-appearing humans, even if transiently.

The human gut is semi-permeable. It is composed of one layer of intestinal epithelial cells, which constitute a physical boundary that isolates the bowel cavity from the lamina propria. The Intestinal epithelial cells excrete specific elements as mucin, anti-microbial peptides (AMP), which include defensins, lysosomes, lectins, lipocalins, and cathelicidins that function to prevent any disturbances in the gut (Wehkamp *et al.*, 2008; Koslowski *et al.*, 2010; Maloy and Powrie, 2011). It is believed that the secretion of these elements into intestinal crypts limits contact between the microbiome and epithelial cells, and subsequently, the lamina propria (Wehkamp *et al.*, 2008; Maloy and Powrie, 2011). Moreover, the intestinal epithelial cells contain specialised cells, such as microfold cells (M cells) and enteroendocrine cells. The function of M cells is to allow for transport the microbiome from luminal crypts to Peyer's patches. However, the enteroendocrine cells excrete different hormones, such as serotonin that works on the nervous systems in response to harm components produced by bacteria (Schuijt *et al.*, 2013).

Beneath intestinal epithelial cells, the lamina propria carries many immune cells for both innate and adaptive immunity, for example, T cells, macrophages, and dendritic cells. Furthermore, the intestinal epithelial cells and some immune cells such as dendritic cells and macrophages have receptors as known as Toll-like receptors on their surfaces to sense breaking of the boundary of the gut or invasion of the bacteria (**Figure 1-9).**The alimentary canal is an essential location for connecting the microbiome with the immune system as well as home to a large number and complicated community of commensal microorganisms. The balance of intestinal microbiota is requisite for the health of the

intestine. Moreover, at an early stage, the intestinal microbes assist in sculpting the human immune system. The immune system incompleteness in new babies and under germ-free environments lets us know that resident microbiota perform a vital role in shaping the immune system of the host (Ivanov *et al.*, 2009a; Duan *et al.*, 2010).

It has been suggested that an imbalance might cause autoimmune diseases in the commensal microbiota in the gut. The physical barriers of the intestine and the innate and adaptive immune system components of the host function to regulate gastrointestinal homeostasis and to react to a microbiome disturbance in the intestine.

Gut homeostasis requires dynamic crosstalk among commensal microorganisms, intestinal epithelial cells, and the local immune cells while at the same time is thought to have an impact on the production and development of some illnesses (including chronic inflammatory diseases) (Artis, 2008; Hooper and Macpherson, 2010). The leaky intestine idea includes passive motion and infiltration of particles, including food and bacteria through the bowel lining cavity to the lamina propria (Morris *et al.*, 1991). The inflammation is shown in some autoimmune diseases such as rheumatoid arthritis may be induced by the elevated permeability of the bowel wall cavity to the gastrointestinal microbial and their particular substances, particularly, toxins produced by bacteria (MARTÍNEZ-GONZÁLEZ *et al.*, 1994) although this result is not consistent with surveys.

One confusing variable in chronic inflammatory disease investigations is that numerous patients are taking NSAIDs treatments, which are drugs that induce elevated permeability of the intestinal lining, for instance, Aspirin, Ibuprofen, Diclofenac (Bjarnason and Takeuchi, 2009). Morris and co-workers found that the increased permeability of the small gut in patients who carry rheumatoid arthritis or ankylosing spondylitis in comparison with healthy subjects (Mielants *et al.*, 1992). Conversely, Mielants and co-workers observed

elevated permeability of the small intestinal lining of autoimmune diseases patients, for example, Rheumatoid arthritis, ankylosing spondylitis and also elevated gut permeability was shown in ankylosing spondylitis patients not taking NSAIDs treatment, however, cannot exclude NSAIDs utilise as a reason (Morris *et al.*, 1991).

It has not yet been whether blood-borne bacteria are exploiting a viable ecological niche, or whether they are transient residents in the circulatory system (Potgieter *et al.*, 2015).

It has been hypothesised that the microbiome translocation may occur from the gut, oral cavity, and other known sites of dysbiosis, to the circulatory system, and specific microbiome can change immune status in some diseases such as RA, AS, and PA. The microbiome of distant sites may be able to access the circulatory system via various routes. For example, members of the gut microbiome may enter the blood from their place of habitation by three approaches **(See Figure 1-8)**; firstly, via dendritic cells that underlie the epithelium may open tight junctions between epithelial cells, transferring processes inside the lumen that directly sample microbes; lamina propria dendritic cells compromise two different subsets: CD103CX3CR1+ DCs (with characteristics of macrophages, promoting TNF-production and development of Th1/Th17 T cells) and CD103+ CX3CR1 DCs (which include the development of regulatory T cells) (Potgieter *et al.*, 2015; Castillo *et al.*, 2019). Secondly, microorganisms may enter the blood through the inflamed or damaged epithelium with a defective epithelial wall by interaction with antigenic material in underlyingtissue. In this case, TLRs of antigen-presenting cells such as macrophages and dendritic cells are capable of identifying the microbial community. In arthritic conditions, the TLR pathway may amplify the abnormal crosstalk existing between antigen-presenting cells, T cells, and B cells, resulting in the generation of high amounts of pro-inflammatory cytokines, local detection of antibodies including RF and ACPA, and the expansion of autoreactive lymphocytes. After that, persistent immune activation can lead to FLS

hyperplasia, complement activation, and neutrophil recruitment leading to cartilage and bone destruction (Arleevskaya *et al.*, 2019). Finally, through M-cells, which lie on top of Peyer's patches and function as specialised cells providing entrance of microbial components to antigen-presenting cells (Potgieter *et al.*, 2015; Castillo *et al.*, 2019).

Peyer's patches are small masses of lymphoid tissues located in the mucous membrane lining of the gut, are distributed along the small intestines with numbers of 100–200 in people. They contain different immune cells, including dendritic cells, macrophages, T cells, and B cells (Neutra, Frey and Kraehenbuhl, 1996; Kobayashi *et al.*, 2019). They are a significant part of the immune system in the intestine through monitoring intestinal microorganism's communities and inhibiting the growth of pathogenic microbial populations in the gut (Makala, Suzuki, and Nagasawa, 2002; Kobayashi *et al.*, 2019). Antigens from microorganisms in the intestine are absorbed by endocytosis (the cellular process in which substances are brought into the cell) via microfold cells covering the surface of Peyer's patches. These antigens are moved on to the lymphoid tissue, where they are ingested through macrophages and also presented to T lymphocytes, and B lymphocytes (Makala, Suzuki, and Nagasawa, 2002). When presented with pathogenic antigens, lymphocytes trigger the immune response via producing pathogen-specific antibodies such as IgA, turning into pathogen-killing cytotoxic T lymphocytes, and moving through lymphatic vessels to lymph nodes to alert the other cells of the immune system. The human body then prepares a full body-wide immune reaction to the pathogen before it can reach beyond the gut (Makala, Suzuki, and Nagasawa, 2002).

Utilising the K/BxN autoimmune arthritis model showed that Peyer's patches T follicular helper cells were crucial for intestine commensal segmented filamentous bacteria-induced systemic arthritis (Teng *et al.*, 2016; Kobayashi *et al.*, 2019). It determined that segmented filamentous bacteria, via driving differentiation and passage of Peyer's patches T follicular

helper cells into systemic sites, increased systemic T follicular helper cell and auto-antibody responses that exacerbated arthritis (Teng *et al.*, 2016). Segmented filamentous bacteria-induced Peyer's patches T follicular helper cell differentiation by limiting the access of interleukin 2 to CD4+ T cells, thereby enhancing T follicular helper cell master regulator Bcl-6 (master Regulator of the germinal centre reaction and a critical oncogene in B Cell Lymphomagenesis) in a dendritic cell-dependent manner (Teng *et al.*, 2016). These findings demonstrate that intestinal microbiota remotely regulated a systemic illness via driving the induction and egress of gut T follicular helper cells (Teng *et al.*, 2016). Furthermore, *Clostridia*-related Gram-positive bacteria that adhere strictly to Peyer's patches in the gut and can excite the immune response via inducing IgA secretion and stimulating B cells. These microorganisms are essential for the development of autoimmunity in the murine K/BxN arthritis model, and the utilise of antibiotics inhibits the progression of arthritis (Horta-Baas *et al.*, 2017).

In response to bacterial translocation, intestinal epithelial cells secrete chemokines that induce the recruitment of dendritic cells to the mucosa. Once activated mature intestinal dendritic cells can induce and prime mucosal T and B cells, eventually shaping the adaptive mucosal immune system. Following maturation, the T and B cells are moved into the bloodstream and, due to surface expression of the appropriate homing markers, home back to reside inside the lamina propria. Microbial antigens presented to B cells produce a commensal-specific IgA response that helps to prevent the commensals from straying beyond the intestinal mucosa (Wiest, Lawson and Geuking, 2014).

Moreover, the oral microbiome could invade the blood circulation as a consequence of chewing, after tooth brushing, or following dental procedures such as periodontal probing or ultrasonic scaling (Kinane *et al.*, 2005; Forner *et al.*, 2006). It is believed that the oral microbiota colonises an assortment of oral surfaces, including gingival cleft fissures and

smooth surfaces, resulting in dental plaque formation (Loyola-Rodriguez *et al.*, 2010). On contact with this tissue-based immune system, it causes tissue cell mobilisation and producing IL-1, IL-6, and TNF-a, following tissue damage by activating matrix metalloproteinases (MMPs) production (a group of enzymes which act to degrade helical fibrillar collagen found in teeth, cartilage, and bone) (Loyola-Rodriguez *et al.*, 2010). Therefore, it is plausible that the oral microbiome might reach distant locations of the human body. Besides, the skin is extensively colonies and is susceptible to incision and consequently represents a large surface area through that such microbes might translocate into the blood circulation system (Depcik-Smith, Hay and Brecher, 2001).

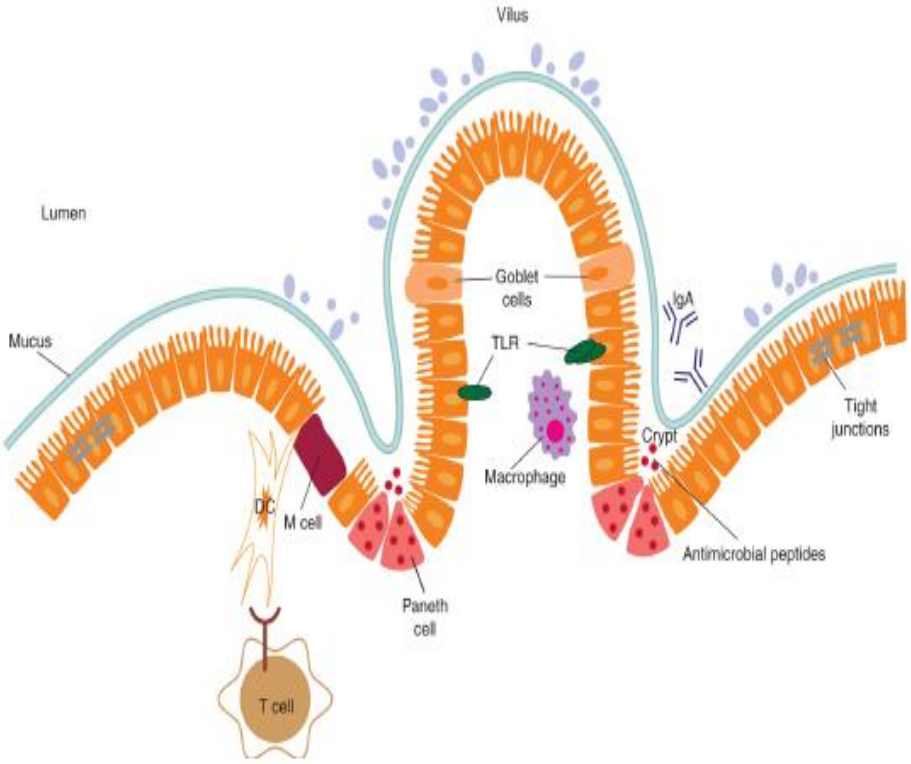


Figure 1-9 Intestinal epithelial cells are packed together, making a tight junction, and controlling the flow of luminal substances. These cells excrete specific elements such as mucin, anti-microbial peptides (AMP), which include defensins, lysosomes, lectins, lipocalins, and cathelicidins that are significant to prevent any disturbances in the alimentary canal. It is believed that the secretion of these particles into intestinal crypts is brought about to limit the contact of microbiome inside epithelial cells and to inhibit arriving the gastrointestinal microbiota from the intestinal lumen to epithelial cells and lamina propria. Beneath intestinal epithelial cells, the lamina propria carries many immune cells for both innate and adaptive immunity, for example, T cells, macrophages, and dendritic cells. Furthermore, the intestinal epithelial cells and some immune cells such as dendritic cells and macrophages have receptors as known as Toll-like receptors on their surfaces to sense breaking of the boundary of the gut or invasion of the bacteria (Costello *et al.*, 2013).

1.4.4.4 Microbiome in other tissue sites

The pulmonary mucosa comprises its own set of microbiotas that may be altered in illness conditions. Importance in the role of the pulmonary microbiota has newly risen in RA (Segal *et al.*, 2013). It has been proposed that, by continuous exposure to bacterial antigens, the respiratory tract may be the potential location of the first events that promote RA initiation and development (Karlson and Deane, 2012; Catrina *et al.*, 2014). Distal airway microbiome dysbiosis was identified in treatment-naive patients with RA, and the results similar to those seen in sarcoid lung inflammation. This population disorder, which is associated with systemic and local autoimmune/inflammatory alterations, could be yet another possible initiating cause for rheumatoid arthritis in some instances (Scher *et al.*, 2016).

The urogenital tract microbiome dysbiosis was also observed by Ebringer et al. noted that the increased isolation rate of *P. mirabilis* from the urine of the female more than male patients with RA and in RA patients as a whole in comparison with healthy individuals (Ebringer and Rashid, 2006).

Under normal physiological conditions, the synovial space is expected to be sterile. The existence of microorganisms results in a septic arthritis diagnosis, a disease considered as a real medical emergency (Ross, 2017). Several studies have been noted that bacteria or bacteria-derived products were identified in the synovial fluid from patients with RA and healthy people (Garrard *et al.*, 2001b; Moen *et al.*, 2006; Martinez-Martinez *et al.*, 2009; Ogrendik, 2009; Témoin *et al.*, 2012b; Reichert *et al.*, 2013). Interestingly, these studies predominating identify the most the microorganisms originated from the oral cavity, and these observations are accompanied via proofs showing the existence of DNAs of these

microorganisms and/or antibodies directed against the originating organism in the circulatory system (Moen *et al.*, 2003, 2006; Lundberg *et al.*, 2008; Martinez-Martinez *et al.*, 2009; Ogrendik, 2009; Hitchon *et al.*, 2010). Based on these findings, bacteria / bacterial nucleic acid may spread the synovial space by blood (as appeared by its concurrent existence in synovial fluid and blood fluids). Moreover, the inflammatory environment of the synovial tissues may help the trapping of these bacterial DNA and increase their apparent concentration in this site (Moen *et al.*, 2006).

Recently, the study by Zhao et al., 2018) has been noted that the existence of genera *Bacteroides* and *Porphyromonas* in both synovial tissues and synovial fluid of rheumatoid arthritis and osteoarthritis samples. Further, *Comamonas, Kocuria, Agrobacterium, Rhodoplanes,* and *Meiothermuswere* are abundant in RA synovial tissues. However, *Bacteroides uniformis, Phascolarctobacterium, Rhodotorula mucilaginosa, Atopobium, Rothia, Turicibacter, Megasphaera, Haemophilus parainfluenzae, Leptotrichia, Bacteroides fragilis, Streptococcus, and Porphyromonas* were dominant in synovial tissues of osteoarthritis. Moreover, the abundance of *Prevotella copri, Haemophilus parainfluenzae, Veillonella dispar, and Treponema amylovorum* increased in synovial fluid of rheumatoid arthritis relative to osteoarthritis synovial fluid; however, the increase of *Bacteroides caccae* was detected in the synovial fluid of osteoarthritis compared with Rheumatoid arthritis (Zhao *et al.*, 2018a).

This study proves the presence of bacterial DNA in synovial and synovial tissue samples from RA and osteoarthritis lesions and reveals potential correlations with the degree of illness. RA and osteoarthritis are discriminated via progressive destruction of cartilage and bone plus dysregulation of synovial function (Témoin *et al.*, 2012a). Osteoarthritis arises from the damage of articular cartilage caused by physical injury and is consequently affected via a variety of intrinsic (for instance, genetic, immunologic, or cellular) agents

(Témoin *et al.*, 2012a). The osteoarthritis synovial layer also shows an inflammatory component, although less notable than in RA (Témoin *et al.*, 2012a).

A common pathological characteristic of RA and osteoarthritis is a synovial inflammatory response (Zhao *et al.*, 2018b). In early arthritis, it is difficult to clinically differentiate between RA and OA using histopathological and imaging techniques (Zhao *et al.*, 2018b). Therefore DNA fragments of the microbiome that assembles in the synovial offer a differentiating characteristic between synovial tissues of osteoarthritis and synovial tissues of RA.

1.4.5 **Mouse studies in Autoimmune/inflammatory disorders**

The mouse is prevalent in human research because they are low-priced, has a short life span, and is anatomically similar to human beings in several respects, particularly the intestinal tract.

Numerous animal studies have observed that intestinal microbiota functions a substantial role in arthritis evolution. It has been revealed that a correlation between certain bacterial species with the evolution of specific T-cell types. Segmented filamentous bacteria (SFB), the gut microbiota of animals such as rodents, were observed to be a potent inducer of Th17 cells in the lamina propria of the mice gut (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009a). Ivanov and his colleagues (Ivanov *et al.*, 2009a) demonstrated that the insertion of segmented filamentous bacteria into germ-free K/BxN mice brought about the elevation of Th17 cells in the intestinal mucosa of the gut. In the mice intestine, the existence of SFB has appeared to induce the evolution of Th17 cells, which has the critical

role in autoimmune illnesses, for example, arthritis (Wu *et al.*, 2010), experimental autoimmune encephalomyelitis (Lee *et al.*, 2011), and colitis (Stepankova *et al.*, 2007). These results propose that specific gut microbiota is capable of promoting a particular subclass of th-cell that resulted in the evolution of autoimmune arthritis.

Further, it has been shown that SKG mouse strains did not evolve arthritis under a germ-free (GF) environment (Wu *et al.*, 2010); however, they develop arthritis into conventional conditions. Moreover, the colonisation of germ-free SKG mice with *P. copri* and a fungal injection were sufficient to simulate arthritis (Maeda and Takeda, 2017).

SKG mouse strains, a new genetic model of RA, in which arthritis development is attributed to a missense mutation in the T cell receptor signalling adaptor molecule Zeta-chain-associated protein kinase (ZAP-70) (a protein generally expressed near the surface membrane of T cells), leading to a defective adverse selection in the thymus and the secrete of autoreactive T cells (Maeda and Takeda, 2017).

Interleukin-1 receptor antagonist knock-out mice observed autoimmune T cell-mediated arthritis by exposure to a specific pathogen-free environment. At the same time, they did not develop arthritis when housed in a germ-free condition (Rogier *et al.*, 2017). However, the colonisation of commensal strains of the genus *Lactobacillus* in these mice prompted arthritis by stimulation of Toll-like receptor (TLR2 and TLR4) (Immune receptors found on the membranes of leukocytes such as dendritic cells, macrophages which function a vital role in the innate immune system) (Rogier *et al.*, 2017).

1.4.6 **16S ribosomal RNA**

16S ribosomal RNA (rRNA) is a component of prokaryotic DNA that exists in all bacteria and Archaea. A 16S rRNA sequence is utilised to distinguish between microorganisms across all main phyla of bacteria and to classify strains down to species level (Woese, 1987). The using of 16S rRNA gene sequences to investigate bacterial taxonomy and phylogeny has been via far the common housekeeping genetic marker utilised for several causes. These reasons involve (i) its existence in all bacteria; (ii) the role of the 16S rRNA gene over time has not altered, proposing that random sequence variations are a more accurate measure of time (evolution); and (iii) the size of 16S rRNA gene is large sufficient for informatics purposes (Janda and Abbott, 2007).

Bacterial 16S rRNA genes comprise nine "hypervariable regions" (V1 – V9) that show significant sequence variety among various bacteria**.** The 16S rRNA V4 gene has been highly recommended as the gold standard for profiling of human microbiome such as the gut, blood and others via several studies (Qin *et al.*, 2010; Lozupone *et al.*, 2013; Santiago *et al.*, 2016; Gloor *et al.*, 2018; Wu *et al.*, 2018; Whittle *et al.*, 2019).

16S rRNA gene sequence analysis is a powerful technique for finding and characterisation members of microbial populations. The bacterial16S gene is ~1,550 bp long, which contains variable nucleotide sequences that are genus- or species-specific and highly conserved. PCR primers targeting the conserved nucleotide sequences of rRNA amplify variable regions of the 16S gene (Relman, 1999). Bacterial communities can be recognised through sequences analysis of the PCR products followed via comparing these sequences with identified sequences found in a database (Clarridge, 2004).

The technique is proper for utilising where the range of pathogens expected to exist is wide and where microorganisms' specific PCRs are unsuitable. Further, it can be utilised to identify bacteria that are difficult to grow or be applied to samples of post-antibiotic therapy (Relman and Falkow, 1992; Brouqui and Raoult, 2001; Harris *et al.*, 2002). Others have utilised this approach on joint fluid, cerebrospinal fluid, tissue, and pus (Hartley and Harris, 2003).

1.4.7 **ITS2**

The internal transcribed spacer 2 (ITS2) region of nuclear ribosomal is considered as a marker to characterise the composition and diversity of fungal populations because it has a number of valued characteristics, for instance, the ease of its amplification, sufficient variability to identify even closely related species, and the availability of conserved regions for designing universal primers (Baldwin *et al.*, 1995). The utilising of the Ribosomal DNA gene to identify fungal populations is depended on the finding of conserved sequences in 5.8S rDNA and 28S rDNA, which can amplify the ITS2 region between those two genes. PCR with fungal-specific primers that target the conserved sequences of 5.8S and 28S rDNA result in the respective amplification of specific regions of the species that vary in length and sequence of the amplicon in according to the species (Turenne *et al.*, 1999). ITS2 of the fungal population were reported to be useful for the identification and characterisation of medically necessary fungal composition which are isolated from human clinical samples such as blood, synovial fluid, stool, oral smears, vaginal swab, skin, and rectal swab (Park *et al.*, 2000; Carr *et al.*, 2005; Huang *et al.*, 2006; Hrabovský and Siegfried, 2009; Nica *et al.*, 2010; Liu, 2011; Gade *et al.*, 2013; Op De Beeck *et al.*, 2014; Allender *et al.*, 2018; Jayasudha *et al.*, 2018).

1.4.8 Potential mechanisms of microbial populations in RA pathophysiology

The involvement of microbes in the pathophysiology of RA is supported through several complementary pieces of evidence from independent reports, and many of them propose a potential incriminatory role of the microbial papulations from classical niches such as the gut, oral and others in the pathogenesis of RA.

Potential mechanisms linking intestinal microbial community and RA include:

1) Microbial translocation from the gut niche into the bloodstream, a process termed atopobiosis, followed by killing these microorganisms by an immune cell known as a phagocyte leaving only the DNA following that the microbial nucleic acid may reach the synovium through the blood (as evidenced by the simultaneous presence in both fluids) (Hernandez, 2017). With relevance to this approach, it is well confirmed that the immune system could distinguish self from non-self-nucleic acid (DNA and RNA) in the human blood and synovial fluid via specific pattern recognition receptions (Chi and Flavell, 2008) and that TLR9 recognises foreign DNA, and RNA is identified via TLR3 on immune cells, producing in the upregulation of various pro-inflammatory cytokines such as TNF alpha, and IL-6, which are associated with RA (Atianand and Fitzgerald, 2013; Castañeda-Delgado et al., 2017).

2) Microbial translocation is followed by a state of dormancy due to activation of the immune system or to unfavourable environmental conditions. With importance to this scenario, dormant microorganism's cells or viable-non culturable bacteria may colonise the joints of RA patients, unidentified by routine culture, whilst retaining the ability to shed inflammatory agents such as lipopolysaccharide (LPS) and other antigenic components

(Pretorius et al., 2017). The circulating LPS is best understood in terms of its effect on bone and joint health. Lipopolysaccharide is an endotoxin from the cell wall of gram-negative bacteria. Through activation of immune cells of the host, pro-inflammatory factors are formed, and these can lead to severe inflammation (Nibali and Henderson, 2016). Pro-inflammatory signalling pathways are induced via binding to specific surface receptors (e.g., TLR4) (Nibali and Henderson, 2016). This will activate multiple signalling pathways (such as nuclear factor-κB (NF-κB), the phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (Akt), and mitogen-activated protein kinase (MAPK) (Nibali and Henderson, 2016). The NF-kB is activated in synovial fluid, which plays a vital role in cartilage damage (Nibali and Henderson, 2016). NF-kB is involved in the control of many genes that are generally activated during adhesion, cell cycle, apoptosis, infection, survival, and in the inflammation process and, therefore, in the production of TNF-α, IL-1β, IL-6, matrix metalloproteinases, and cyclooxygenase-2 (COX-2) (Nibali and Henderson, 2016).

3) Immune cells at the gut endothelium are in constant contact with the gut microbiota. Dendritic cell processes extending through the gut endothelium respond to the presence of the gut microbiota through secretion of factors that lead to changes in gut microbial populations and other immune cells (Hernandez, 2017). Additionally, microbes within the gut secrete factors such as short-chain fatty acids that can mediate the activity of T cells, thereby reducing local inflammation. Immune cell response to the gut microbiota may lead to the secretion of pro- or anti-inflammatory factors and/or the migration of activated immune cells to the systemic circulation and eventually to the bone and the synovium (Hernandez, 2017).

The oral microbiome can enhance immunological reactions via several mechanisms. The oral microbiome can invade the blood circulation as a consequence of bacteremia

(microbiome presence in the bloodstream), and this frequently occurs the following chewing or after tooth brushing and dental procedures such as periodontal probing or ultrasonic scaling (Kinane *et al.*, 2005; Forner *et al.*, 2006). It is believed that the oral microbiota colonises an assortment of tooth surfaces, including gingival cleft fissures and smooth surfaces, resulting in dental plaque formation (Loyola-Rodriguez *et al.*, 2010). In contact with this tissue-based immune system, it causes tissue cell mobilisation and producing IL-1, IL-6, and TNF-a, following tissue damage by activating matrix metalloproteinases (MMPs) production (Loyola-Rodriguez *et al.*, 2010). Therefore, it is plausible that the oral microbiome might reach distant locations such as the blood and synovium of the human body and be implicated in the pathogenesis of RA.

Moreover, another mechanism is used by some oral microbiota, such as P*. gingivalis.* It is a Gram-negative bacterium that is found in the gingival clefts of people who induce an inflammatory response in the gum as well as in healthy people. This microorganism has the capacity to release peptidyl arginine deiminase (PAD) that brings about the citrullination that can excite the immune system to release autoantibodies such as ACPAs, which take part in the pathogenesis of RA (du Teil Espina *et al.*, 2019).

The urinary microbial community can enhance immunological reactions in RA via *Proteus species*. It has been found that a “shared epitope” EQR (K) RAA shows “molecular mimicry” with the related sequence ESRRAL found in *Proteus* hemolysis (Pretorius *et al.*, 2017). Further, *Proteus* urease contains a sequence IRRET, which has “molecular mimicry” with the related LRREI found in collagen XI of hyaline cartilage (Pretorius *et al.*, 2017). Besides, *Proteus* sequences in hemolysin and urease as well as the self-antigens, HLA-DR1/4 and collagen XI, each contain an arginine doublet, thereby providing a substrate for peptidyl arginine deiminase to give rise to citrulline, which is the main

antigenic component of CCP, antibodies to which are seen in early cases of RA (Pretorius *et al.*, 2017).

It was revealed that the accumulation of supraglottic pathogens (e.g., Prophyromonas, Prevotella sp) in the lungs were associated with airway inflammation. Perhaps the lung is thus involved in the process of citrullination and thus autoimmunity in RA (Nibali and Henderson, 2016).

1.5 Aims and Objectives

Research *aims* are to:

1- To investigate the association of blood dysbiosis with rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis diseases.

2- To examine the association of dysbiosis in synovial fluid with rheumatoid arthritis.

3- To link dysbiosis in multiple microbiome niches in the collagen-induced arthritis model following the induction of experimental RA.

The project approach is to use a combination of human patient samples with diseases and a mouse model of collagen-induced arthritis. This aim will be achieved through the following three objectives:

1- Characterisation of the blood microbiome of patients with rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, relative to a number of healthy control subjects.

2- Characterisation of the synovial fluid microbiome of patients with rheumatoid arthritis in comparison to healthy control subjects.

3- Characterisation of the gut, synovial fluid, urine, blood, and serum microbiome of mice with collagen-induced arthritis (CIA) and healthy controls subjects.

Chapter 2

2 Materials and methods

2.1 Overview

The methods and materials delineated in this section here were used in all studies detailed in this research. Any particular or different approaches that have been applied to specific analyses will be explained in the related chapters.

2.2 Samples

The following methods were applied to a series of studies including 1- Molecular characterisation and immunological analyses of the bacterial and fungal microbiome in the blood of RA both prior to and following treatment, ankylosing spondylitis, psoriatic arthritis, and healthy control subjects 2- Molecular characterisation and immunological analyses of the bacterial and fungal microbiome in the synovial fluid of rheumatoid arthritis patients and healthy control subjects 3- Molecular characterisation of the gut, synovial fluid, urine, blood, and serum of the bacterial microbiome in collagen-induced arthritis and controls mice subjects.

2.2.1 **Human blood samples**

Serum samples from twenty rheumatoid arthritis patients were used for microbiome characterisation and immunological analyses. Samples were collected both before (RA V0) and three months following (RA V3) the instigation of treatment. Samples of serum were collected at the Haywood Hospital Trust under NREC 16/LO/0957, IRAS Project ID 198240. Clinical details of RA patients are shown in **Table-2.1**.

In addition to the RA patient serum, four plasma samples from patients with RA, four serum samples from ankylosing spondylitis (AS) patients, four serum and plasma samples from psoriatic arthritis (PA) patients, and four serum and plasma samples from healthy control subjects who were free from disease, were obtained to allow comparison among the different disease states. Clinical details of patients and healthy individuals are seen in **Table-2.2**. RA plasma, AS serum, PA plasma, and serum, as well as healthy control serum and plasma samples were obtained from Sera Laboratories Limited, UK. The authors obtained ethical approval (Keele University ERP3) and written informed consent to utilise the samples for the research reported herein. For plasma collection, whole blood was drawn, following alcohol cleansing of the skin surface, into EDTA containing tubes and stored on ice before centrifugation at 3,000 rpm for 5 minutes to obtain the plasma component following the samples were collected in fresh tubes and stored at -80$_{o}$C. However, for serum collection, whole blood was drawn into fresh tubes and allowed to sit at room temperature for at least 30 minutes to ensure clotting and then centrifuged at 3,000 rpm for 5 minutes to obtain serum. The serum was frozen at -80°C. After that, these samples were sent to Keele University for analysis. Preparation of the PCR reactions was carried out in sterile conditions applying an ultraviolet (UV) bench-top hood to decrease the chance of the reaction tubes and PCR reagents being exposed to contamination with

microorganisms, which present in the immediate environment. UV germicidal irradiation was also carried out by exposing the PCR tubes, PCR workspace, pipettes, PCR master mix, and molecular biology grade water to short-wavelength UV for 30 mins before use.

Further, to having sterile environments and using UV germicidal irradiation, a negative control reaction (in which a human biofluid sample was replaced with UV-irradiated nuclease-free water) was included in each experiment to confirm that none of all reagents was contaminated with target DNA.

Table 2-1 Clinical details of patients with RA are obtained from Haywood hospital

STUDY ID	RF/CCP status	Drugs
RA 135	Positive	MTX monotherapy
RA 138	Negative	MTX/SSZ/HCQ
RA 109	Negative	MTX monotherapy
RA 140	Negative	MTX monotherapy
RA 150	Negative	MTX/SSZ/HCQ
RA 151	Positive	MTX
RA 145	Positive	MTX/SSZ/HCQ
RA 139	Negative	SSZ/HCQ
RA 146	Positive	MTX/HCQ
RA 111	Negative	MTX/HCQ
RA 141	Positive	MTX/SSZ/HCQ
RA 115	Negative	MTX/SSZ/HCQ
RA 143	Negative	MTX/SSZ/HCQ
RA 103	Positive	MTX/HCQ
RA 113	Positive	MTX
RA 128	Positive	MTX/SSZ/HCQ
RA 116	Negative	MTX/SSZ/HCQ
RA 144	Negative	MTX/SSZ/HCQ
RA 112	Positive	MTX
RA 107	Negative	MTX

RF – Rheumatoid Factor, CCP – Anti cyclic citrullinated peptide, MTX – methotrexate, SSZ – sulphasalazine, HCQ - hydroxychloroquine

Table 2-2 Clinical details of patients and healthy individuals are collected from sera Lab Company

ID	Samples	Gender	Age	Diagnosis	Medications
BRH1095328	Plasma	Male	73	Rheumatoid Arthritis (RA)	Tramadol 50mg, Vitamin D 2000u, CA 1000mg
BRH1095329	Plasma	Male	49	Rheumatoid Arthritis (RA)	Methotrexate 20mg, Folic Acid 1mg, Naprosyn 375mg
BRH1095330	Plasma	Female	66	Rheumatoid Arthritis (RA)	Methotrexate 15mg, Folic Acid 1mg
BRH1095331	Plasma	Female	73	Rheumatoid Arthritis (RA)	Methotrexate 20mg, Folic Acid
BRH1090908	Plasma	Male	54	CONTROL	NONE
BRH1090909	Plasma	Female	59	CONTROL	NONE
BRH1090910	K3 EDTA Plasma	Female	53	CONTROL	NONE
BRH1090905	Serum	Male	63	CONTROL	NONE
BRH1090906	Serum	Male	62	CONTROL	NONE
BRH1090903	Serum	Female	53	CONTROL	NONE
BRH1090904	Serum	Female	62	CONTROL	NONE
BRH1095340	Serum	Female	38	Ankylosing Spondylitis	Methotrexate
BRH1095341	Serum	Male	63	Ankylosing Spondylitis	Methotrexate
BRH1095342	Serum	Female	34	Ankylosing Spondylitis	Methotrexate, Enbrel
BRH1095343	Serum	Male	38	Ankylosing Spondylitis	Methotrexate; Enbrel
BRH1095344	Plasma	Female	44	Psoriatic Arthritis	Lisinopril-Hydrochlorothiazide 20-25mg, Amlodipine Besylate 5mg.
BRH1095345	Plasma	Female	57	Psoriatic Arthritis	Enbrel 50mg/ml, Ibuprofen 600mg, Vicodin 7.5-750mg, Zithromax 250mg, Cephalexin 500mg,
BRH1095346	Plasma	Male	68	Psoriatic Arthritis	Aspirin 81mg, Enbrel 50mg
BRH1095347	Plasma	Male	71	Psoriatic Arthritis	Coreg 3.125mg, Zetia 10mg, Atorvastatin 40mg
BRH1095348	Serum	Female	69	Psoriatic Arthritis	Januvia 100mg, Metoprolol 25mg, Potchlorer tabs 10mg, Chlordiaz/Clidinium Liborax 5/2.5
BRH1095349	Serum	Male	30	Psoriatic Arthritis	Otyla 30mg, Lialda 4.8g
BRH1095350	Serum	Female	61	Psoriatic Arthritis	SSZ
BRH1095351	Serum	Male	41	Psoriatic Arthritis	Dovonex

2.2.2 **Human synovial fluid samples**

Synovial fluid samples from sixteen Rheumatoid arthritis patients, and nine sex and BMI-matched disease-free individuals were obtained for microbiome characterisation and immunological assessment. Synovial fluid was obtained via aspiration of the affected or healthy knee synovial fluid utilising a sterile needle and then transferred to a sterile microcentrifuge tube and stored at -80$_{o}$C before further analysis. Samples were obtained from Sera Laboratories Limited. Features of the RA patients and healthy population are shown in **Table-2.3.** The Independent Investigational Review Board Inc. (BRI-0722) ethically approved sample collection by Sera Laboratories Limited from human donors giving informed written consent. Additionally, the ethical approval from Keele University Ethical Review Panel 3 was obtained for the study reported herein. All experiments were carried out according to relevant guidelines and regulations.

Table 2-3 Features of RA patients and healthy population are taken from Sera Lab Company

Patient ID#	Gender	Age	Diagnosis
1339	Female	65	RA
1340	Female	67	RA
1341	Female	67	RA
1342	Female	67	RA
1343	Female	70	RA
1344	Female	69	RA
1345	Male	56	RA
1346	Male	52	RA
1347	Male	55	RA
1348	Male	66	RA
1349	Male	74	RA
1350	Male	69	RA
BRH1095336	Female	67	RA
BRH1095337	Female	67	RA
BRH1095338	Male	67	RA
BRH1095339	Male	67	RA
1351	Female	57	Healthy
1352	Male	64	Healthy
1353	Male	64	Healthy
1354	Male	53	Healthy
1355	Male	50	Healthy
1356	Male	74	Healthy
1357	Female	64	Healthy
1358	Female	55	Healthy
1359	Female	68	Healthy

2.2.3 **Mice biological samples (stool, synovial fluid, urine, blood, and serum)**

Twenty DBA/1J mice aged eight weeks were procured from Charles Rivers (UK) and acclimatised for seven days before study commencement. DBA/1J mice were housed up to 5 per cage in an individually ventilated cage with individual mice recognised through the tail marker. All mice were provided free access to a standard approved commercial diet and sterilised water throughout the study. The laboratory room was prepared under optimal conditions: 20-24°C, 12h light/dark cycle, and 40-70% humidity. Male DBA/1 mice (n=10) were injected with bovine type II collagen (Chondrex Cat No. 20021) in Complete Freund's adjuvant (50 µl of 2 mg/ml emulsion with Complete Freund Adjuvant) (Chondrex Cat No. 7009) intra-dermally in the tail on Day 1 and Day 21.

Mean animal bodyweight, arthritic index, and hind paw thickness were checked three times per week. For control animals (n=10), Phosphate-buffered saline (PBS) was injected instead of bovine type II collagen.

This work was performed by Axis Bioservices (UK), including the collected mouse samples and the indication RA in mouse subjects. All protocols to be used in generating arthritis have been approved by the Axis Bioservices Animal Welfare and Ethical Review Board, and all procedures are carried out under the guidelines of the Animal (Scientific Procedures) Act 1986.

The arthritic Index was measured for each animal three times per week during the study. This system is based on evidence of erythema and swelling with values indicative of the following:

• Grade 1: mild, with skin slight redness or redness, and swelling in 1-2 toe joints

• Grade 2: moderate, with slight redness and swelling in the feet, ankles, or foot pads

• Grade 3: severe, with redness and significant swelling of the toes, feet, and joints

• Grade 4: severe, with highly swollen and red feet, toes, and joints, in addition to stiffness and deformity (Zhang et al., 2019).

As expected, control animals had an arthritic index of 0, indicative of no inflammation observed.

2.2.3.1 Biological samples from the mouse study

2.2.3.1.1 Blood Collection

On sampling day (18 days posted the second injection when inflammation was satisfactory), animals were euthanised via carbon dioxide. Blood was collected via cardiac puncture. 250 µl blood was placed into tubes coated with lithium heparin to prevent blood clotting and stored at -20°C before shipment. The remaining blood was placed into an Eppendorf, allowed to sit at room temperature for at least 30 minutes to ensure clotting, and then centrifuged at 3,000 rpm for 5 minutes to obtain serum. The serum was frozen at

-20°C before shipment to Keele University on dry ice. Samples obtained are from individual mice and are not pooled.

2.2.3.1.2 Synovial fluid collection

On sampling day (18 days post the second injection), synovial fluid was collected via fine needle aspiration by locating the space between the paella and tibia and gently removing the fluid. Synovial fluid was flash-frozen and shipped to Keele University on dry ice. Samples obtained are from individual mice and are not pooled.

2.2.3.1.3 Urine and faeces collection

On days 18 post second injection, urine, and faeces were collected via an adapted specimen collection cage. Samples were flash-frozen and shipped to Keele University on dry ice. Samples obtained are from individual mice and are not pooled.

After collected faeces, synovial fluid, urine, blood, and serum samples in Axis Bioservices (UK), the samples were sent to Keele and frozen at -80°C.

2.3 Microbiome characterisation

2.3.1 Microbiome characterisation of human blood and synovial fluid samples

Laboratory procedures and experiments of human biological samples are shown in **Figure**

Figure 2-1

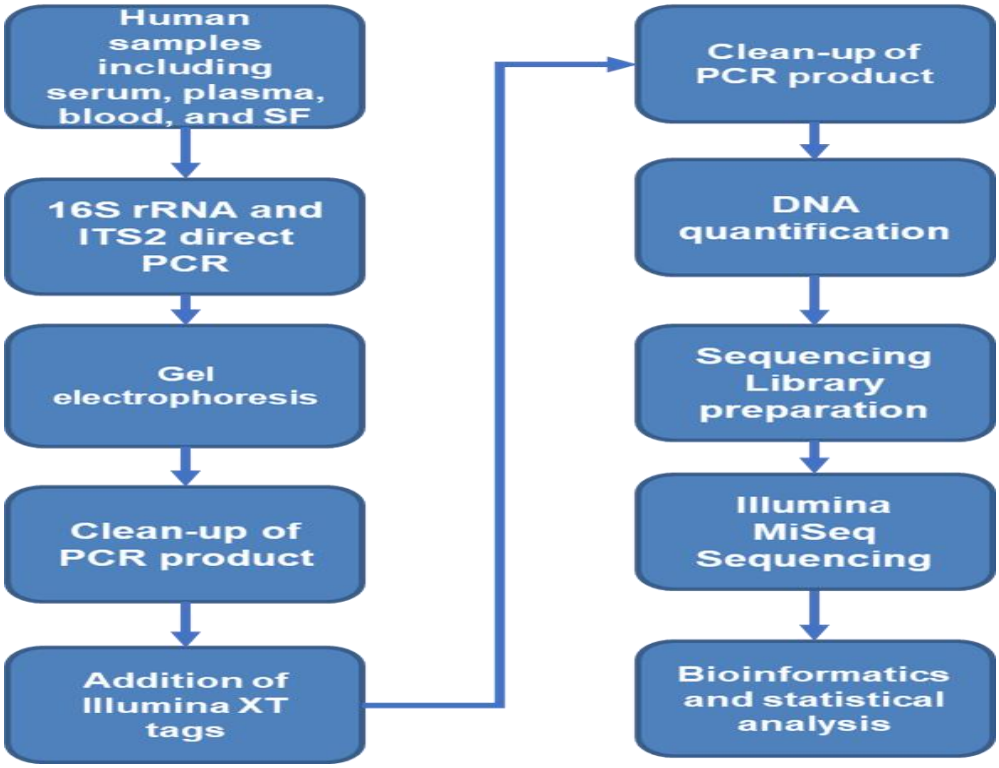


Figure 2-1 Flowchart of the Laboratory procedures and experiments of human biological samples

2.3.1.1 16S rRNA and ITS2 PCR

The bacterial 16S rRNA and fungal ITS2 genes were amplified through direct PCR utilising the primers listed in **Table 2-4**. Direct PCR does not require a separate DNA extraction step, which has previously been suggested as a source of contaminants that affect all downstream applications.

A first round of PCR of 16S V4 and ITS2 regions was carried out with four microliters of each human biofluid sample as the template in a final volume of 20 µl, which contained 10 µl Phusion blood PCR buffer (Thermofisher), 0.4 µl (2 U) Phusion blood DNA polymerase, 1µl of each primer (10uM) of bacterial 16S rRNA (16SV4_F and16SV4_R) and fungal ITS2 genes (ITS2_F and ITS2_R), and 3.6 µl of UV-irradiated nuclease-free water. A negative control reaction (in which a human biofluid sample was replaced with UV-irradiated nuclease-free water) was included in each experiment to confirm that none of all reagents was contaminated with target DNA. A positive control (E Coli as the template) was also prepared in each experiment to ensure successful PCR amplification. The approximate amplicon sizes of first PCR for 16S V4 is ~295 bp (Ziesemer *et al.*, 2015; Whittle *et al.*, 2019), and ITS2 ranged between 245-595bp (Bokulich and Mills, 2013; Hoggard *et al.*, 2018).

16S V4 region was amplified with Schloss V4-F/V4-R via a touchdown PCR program, as follows an initial denaturation step performed at 98°C for 5 minutes, followed by 35 cycles of denaturation (98°C, 10 seconds), annealing (55°C, 5 seconds) and elongation (72°C, 15 seconds), and a final extension of 7 minutes at 72°C. The protocol for the ITS2 gene amplification utilising ITS2-F/ ITS2-R was the same as utilised for the 16S rRNA PCR except increase the extension time from 15 seconds to 45 seconds.

Table 2-4 Primers used in this study

Primer Name	Primer Sequence (5' – 3')	Length	Annealing temperature
16SV4_F	GTGCCAGCMGCCGCGGTAA M= A or C	19	
16SV4_R	GGACTACHVGGGTWTCTAAT H= A or C or T V= A or C or G W= A or T	20	55°C
16SV4_XT_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGGTGCCAGCMGCCGCGGTAA	52	
16SV4_XT_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGGACTACHVGGGTWTCTAAT	54	55°C
ITS2_F	GCATCGATGAAGAAC GCAGC	20	
ITS2_F	TCCTCCGCTTATTGATATGC	20	55°C
ITS2_XT_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AG GCATCGATGAAGAACGCAGC	53	
ITS2_XT_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG TCCTCCGCTTATTGATATGC	54	55°C

2.3.1.2 DNA Gel electrophoresis

Gel electrophoresis of 5ul of each PCR product was utilised to confirm successful amplification. Briefly, a 2% w/v agarose gel was prepared using four agarose tablets (Thermo Fisher Scientific, UK) to 100 ml of 1X Tris-acetate-EDTA Buffer (TAE) containing a mixture of Tris base 40 mM, acetic acid 20 mM, and EDTA 1 mM (Thermo scientific, UK) according to manufacturer's protocol and then heating the gel solution in a microwave until the agarose was completely dissolved. The gel solution was poured into a gel casting tray once the mixture was cooled down to about 60°C. One or two gel comb(s) were placed in the tray to produce the wells, and then the mixture was allowed to sit at room temperature for at least 60 minutes.

Following gel preparation, adequate TAE buffer was added to cover the wells, and the tray and the comb were then removed. The loading samples were then made through mixing up 5 µl of the PCR sample with 1 µl of Gel Loading Dye, Purple (6X) (NEB, UK), which contains Ficoll® for brighter, tighter bands, and EDTA to stop enzymatic reactions. Subsequently, samples were applied, and to determine the size of the DNA fragment, a 100 bp DNA ladder with a concentration of 500 µg/ml (consists of 12 blunt-ended DNA fragments between 100 and 1,517 bp) (NEB, UK) was used. The electrophoresis machine was set to run at 85 V for 60min. After that, the DNA was stained by ethidium bromide staining (EtBr) (Sigma, Gillingham, UK) for 15 minutes. In the final step, DNA bands were visualised by the UV transilluminator (G: Box Gel Image Analysis Systems, Syngene).

2.3.1.3 Clean-up of PCR product samples

PCR Purification Kit (Qiagen, cat. no. 28004) was used to purify the remaining PCR product (15µl) from excess primer and PCR reagents according to the manufacturer's protocol. Briefly, one volume of PCR reaction was mixed to five volumes of PB buffer and vortexed. The mixture was applied to a QIAquick spin column that was placed in a fresh 2 ml collection tube and then centrifuged for 1 minute at 13,000g, and the filtrate was discarded. After that, 0.75ml of PE wash buffer was applied to the QIAquick column and then centrifuged for 1 minute at 13,000g, and the filtrate was removed. The final step, 10µl of elution buffer EB was applied to the centre of the QIAquick column membrane, and the column was left to stand for 1 min to absorb all elution buffer inside the column and then centrifuged for 1 minute at 13,000g. The solutions were eluted in 10 µl Buffer EB (10 mM Tris·Cl, pH 8.5). A "kit control" was run together with this procedure and involved the purification of 15µl of UV irradiated nuclease-free water, again to ensure the kit used was free from contaminating DNA. Following the remainder was stored at - 20°C. Following standard agarose gel electrophoresis and staining with ethidium bromide to check to remove the excess primers from amplicons and to ensure from the presence of the resulting PCR products.

2.3.1.4 Confirmation of the absence of All PCR negative reactions

All PCR negative reactions were tested with high sensitivity DNA quantification utilising the Qubit 3.0 hsDNA kit (Invitrogen, UK) to ensure the non-appearance of any products of amplification in these controls. Briefly, the working solution was ready via diluting the Qubit

reagent 1:200 in Qubit buffer. This was initially set as a master mix adequate for a total of mixtures in addition to 2 standard tubes. The individual test tubes were ready in 500 ul PCR tubes according to **Table-2.5** beneath.

Table-2.5 Volumes for preparing a sample and standard test tubes for Qubit 3.0

Volume	Standard test tubes	Sample test tubes
The volume of working solution	190 µl	199 µl
The volume of standard	10 µl	0
The volume of the DNA sample	0	1 µl
Total volume in each test tube	200 µl	200 µl

After that, the test tubes were quickly vortexed and next incubated for two minutes at room temperature. Lastly, they were placed in a Qubit 3.0 Fluorometer to gain a concentration of ng/µl of each sample.

2.3.1.5 Addition of Illumina XT tags

In order to add XT_tagged primers, which help to facilitate Illumina MiSeq library preparation, The second PCR amplification was achieved in a total volume of 50 µl containing 10 µl of 5X Platinum Superfi Buffer, 1µl 10mM dNTP mixture, 0.5µl Platinum Super-Fi polymerase, 2.5 µl of 10 µM each 16SV4_XT_F and 16SV4_XT_R for bacterial 16S or ITS2_XT_F and ITS2_XT_R for fungal ITS2, 5µL from the successful first-round PCR reaction and 38.5 µL of UV-irradiated nuclease-free water. PCR conditions of bacterial 16S and fungal ITS2 were as follows: 98C x 2mins, 7 x cycles, 98C x 10sec, and

72C x 20sec and 72C x 5mins. The approximate amplicon sizes of second PCR for 16S V4 is ~355 bp, and ITS2 ranged between 300–650 bp.

2.3.1.6 Clean-up of PCR product s using AMPure XP magnetic beads

AMPure XP magnetic beads were utilised to purify the amplicon from primer dimers and excess PCR reagents. At a ratio of 0.8, beads to sample (v/v) were added. The mixture was mixed via pipetting and incubated for 1 min at room temperature to secure the binding of PCR products to the magnetic beads. Next quick centrifugation, the sample was put on a magnetic stand to isolate the magnetic beads from the supernatant and left until the solution was apparent. The supernatant was then removed without disturbing the beads, which contained the DNA targets. Following the beads were washed through adding 180 µl of 85% (v/v) ethanol (Sigma, UK) and then incubated at room temperature for 30 seconds prior carefully discarding the ethanol supernatant. The beads were then dried and eluted in 20 µl of UV-irradiated nuclease-free water. The beads were then isolated using the magnetic stand, and the eluted DNA targets were isolated and stored at -20 °C. After that, the amplicons were quantified utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen, UK) as described above.

2.3.2 **Microbiome characterisation of Mice samples**

Laboratory procedures and experiments of mice's biological samples are shown in the flowchart (**Figure 2-2).**

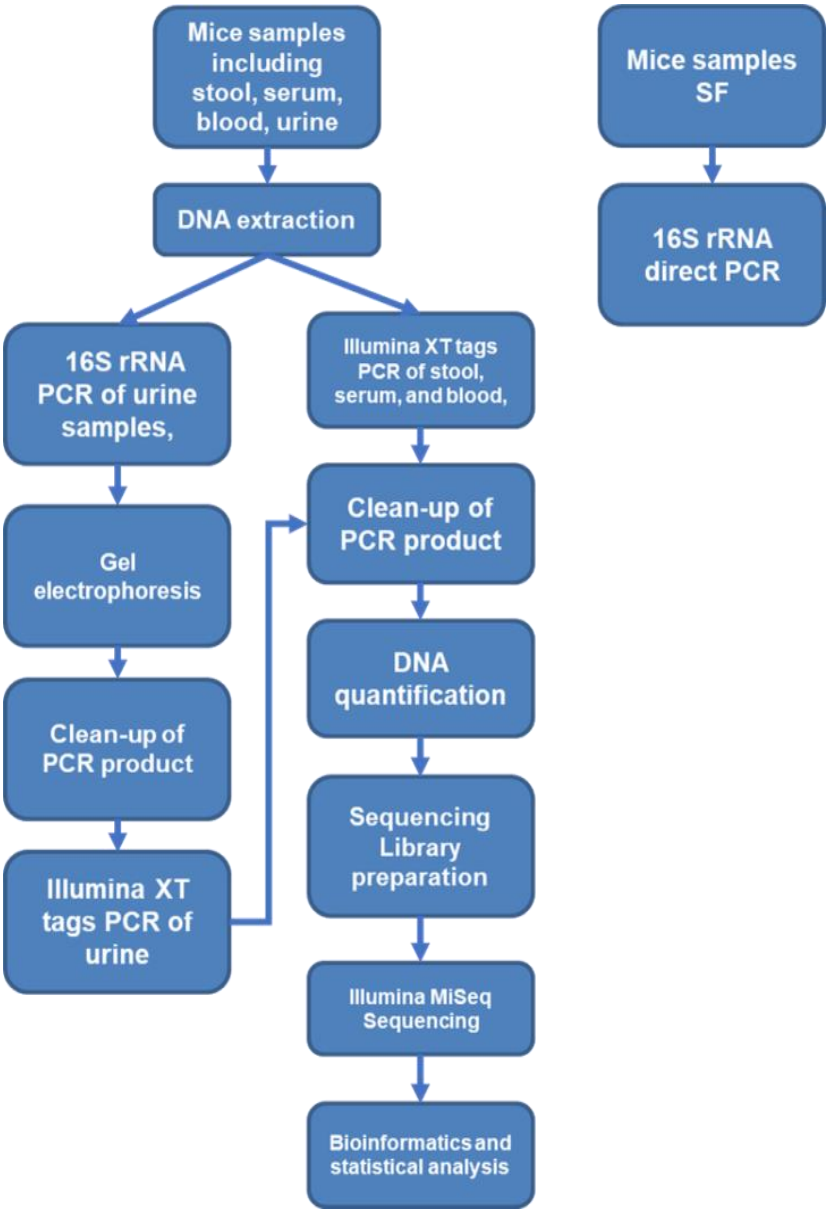


Figure 2-2 Flowchart of the Laboratory procedures and experiments of mice biological samples

2.3.2.1 DNA extraction of Mice samples

A total of three extraction methods were utilised to extract bacterial DNA from mice biological samples which are DNeasy PowerSoil Kit (Qiagen, UK) for stool samples, DNeasy® Blood and Tissue Kit (Qiagen, UK) for blood and serum samples, and PureLink™ Microbiome DNA Purification Kit (Invitrogen) for urine samples.

2.3.2.1.1 DNA extraction from stool samples

DNeasy PowerSoil Kit was used to extract DNA from stool samples. Bacterial genomic DNA was extracted from 250mg mouse stool using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's instructions. Briefly, 0.25 g of mice faecal sample was placed in the dry bead tube, which contains 750 µl of bead solution and briefly shaken. C1 solution was added, the specimens gently vortexed and incubated for 10 minutes at 65°C. Specimens were horizontally shaken for 10 min. Samples were then centrifuged for 1 min at 13,000 × g., the liquid moved to the fresh 2 ml collection tube, and the rest of the protocol continued as recommended via the manufacturer. DNA was eluted in 100 µl by C6 solution and stored at −20 °C.

2.3.2.1.2 DNA extraction from blood and serum samples

DNeasy® Blood and Tissue Kit was carried out to extract DNA from mouse blood and serum samples according to the manufacturer's instructions for the kit. For each sample, 100 ul of mouse blood sample (whole blood and serum) was placed in a clean 1.5 ml microcentrifuge tube. A volume of 20 µl proteinase K was added to each mouse blood sample and modified the volume to 220 µl by PBS. A volume of 200 µl Buffer AL was added and briefly vortexed, and then incubated for 10 min at 56°C. Next, 200 µl ethanol (96%) was added and thoroughly vortexed. A mixture was pipetted into a DNeasy® Mini Spin Columns located in a clean 2 ml collection tube and then centrifuged at 8000 rpm for 1 min, and the collection tube and flow-through were discarded. The DNeasy® Mini Spin Column was transformed into a fresh 2 ml collection tube. Following 500 µl Buffer AW1 was added and then centrifuged at 8000 rpm for 1 min and discarded flow-through and the collection tube. After that, 500 µl Buffer AW2 was added to the mixture, and then the tube centrifuged at full speed for 4 mins. After a volume of 100 µl Buffer, AE was directly pipetted onto the DNeasy membrane. The sample was incubated for 2 mins at room temperature and centrifuged 8000 rpm for 1 min and stored at −20 °C before analysis.

2.3.2.1.3 DNA extraction from urine samples

PureLink™ Microbiome DNA Purification Kit was used to extract DNA from urine samples. Urine samples were centrifuged at 13,000 × g for 10 min, the supernatant was discarded, and the pellets were stored until further processing. 800 µl of S1—Lysis Buffer was added to the pellets and briefly vortexed, and then transferred the mixture to the bead-beating

tube. 100 µl of S2—Lysis Enhancer was added and mixed through the vortex. The sample was then incubated at 65°C for 10 minutes. After that, Specimens were horizontally shaken for 10 min and Centrifuged at 13,000 × g for 2 minutes. 500 µl of the supernatant moved to the clean 2 ml collection tube. Following 900 µl of S4—Binding Buffer was added to the tube and vortexed briefly. The sample mixture was loaded onto a spin column-tube assembly and Centrifuged at 13,000 × g for 1 min, and the filtrate was discarded. The spin column was placed in a clean collection tube, and a volume 500 µl of S5—Wash buffer was added, and then centrifuged at 13,000 × g for 1 min. The flow-through was discarded and then centrifuged the spin column-tube assembly at 13,000 × g for 1 min. The spin column was placed in a clean tube. After a volume of 50 µl of S6—the elution Buffer was directly pipetted onto the spin membrane. The sample was incubated for 1 min at room temperature and centrifuged 8000 rpm for 1 min and stored at −20 °C.

2.3.2.2 Amplification of 16S rRNA gene by Polymerase Chain Reaction

2.3.2.2.1 16S rRNA gene PCR of mice faecal samples

To produce 16S rRNA gene amplicons from mice feces samples, 5µl of DNA was used as a template in a 25µl reaction, with 2.5µl of 10X High Fidelity PCR Buffer, 1µl of 50 mM MgSO4, 0.5 µl of 10mM dNTP mixture, 0.1µl of Platinum Taq High Fidelity polymerase, 0.5 µl of each primer (10uM) targeting 16SV4_F (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT -3') and 16SV4_R (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3'),

and 14.9µl of the nuclease-free water that had been subject to 15 minutes UV-irradiation. Negative and positive control reactions were prepared. Amplification conditions involved an initial denaturation step at 98 centigrade for 5 minutes, followed by 30 cycles at 98 centigrade 15 seconds, 55 centigrade for 5 seconds, with extension for 30 seconds at 72 centigrade. Following electrophoretic separation of 5µl of each PCR product was applied, the remainder (20µl) was purified of primer dimers and PCR components utilizing AMPure XP magnetic beads (Agencourt) at a ratio of 0.8 beads to sample (v/v), eluted in 20µl of UV-irradiated nuclease-free water, and quantified using the Qubit 3.0 high-sensitivity DNA kit (Invitrogen).

2.3.2.2.2 16S rRNA PCR of mice blood samples (whole blood and serum)

The bacterial 16S rRNA was amplified by PCR utilising the primers listed in **(Table 2.1).** First PCR round utilising primers 16SV4_XT_F and 16SV4_XT_R. PCR was carried out in reactions containing 2µl of each DNA sample, 10µl 2X Phusion Master Mix, 1µl of each primer of (10uM) 16S rRNA, and 6 µl of the nuclease-free water that had been subject to 15 min UV-irradiation, in a final volume of 20 µl. Negative and positive control reactions were prepared.

Cycling conditions involved an initial denaturation step at 98°C for 5 min followed by 30 cycles of 30 sec 98°C, 5-sec annealing at 55°C and 30-sec elongation at 72°C, followed by a final extension of 7 minutes at 72°C. Following electrophoretic separation of 5µl of each PCR product was used. The remainder PCR product (15µl) was purified from excess primer and PCR reagents using the QIAquick PCR Purification Kit. A “Kit control” was run

together with this procedure and included the purification of 15µl of UV irradiated nuclease-free water, again to ensure the kit used was free from contaminating DNA.

After this, PCR products were then purified utilising AMPure XP magnetic beads (Agencourt) at a ratio of 0.8 beads to sample (v/v), eluted in 20µl of UV-irradiated nuclease-free water, and quantified utilising the Qubit 3.0 high-sensitivity DNA Kit (Invitrogen).

2.3.2.2.3 16S rRNA PCR of mice urine samples

The first PCR round utilising primers 16SV4_F and 16SV4_R was carried out in reactions comprising 2µl of each DNA sample, 12.5µl GoTaq Green DNA (Promega), 2µl of each primer of (10uM) 16S rRNA, and 6.5µl of the nuclease-free water that had been subject to 15 minutes UV-irradiation, in a final volume of 25 µl. Negative and positive control reactions were prepared.

PCR reactions were carried out using the respective protocol: an initial denaturation step performed at 95°C for 2 minutes, followed by 35 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds), and extension (73°C, 45 seconds), and a final elongation of 5 minutes at 73°C.

Following electrophoretic separation of 5µl of each PCR product, the remainder (20µl) was purified to remove the excess primer and PCR components using the QIAquick PCR Purification Kit. A “Kit control” was run together with this process and involved the purification of 15µl of UV irradiated nuclease-free water, again to ensure the kit used was free from contaminating DNA.

The second round of PCR amplification was completed as described in **(2.3.1.5).** Following PCR products were purified utilising AMPure XP magnetic beads (Agencourt) at a ratio of 0.8 beads to sample (v/v), eluted in 20µl of UV-irradiated nuclease-free water, and quantified utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen).

2.3.2.2.4 16S rRNA PCR of mice synovial fluid samples

To generate 16S rRNA gene amplicons of mice SF samples, 4µl of DNA was used as a template in a 20µl reaction, 10µl Phusion blood PCR buffer (Thermofisher), 0.4µl (2 U) Phusion blood DNA polymerase, 1µl of each primer (10uM) targeting 16SV4_F (GTGCCAGCMGCCGCGGTAA-3'), and 16SV4_R (GGACTACHVGGGTWTCTAAT), and 3.6µl of the nuclease-free water that had been subject to 15 minutes UV-irradiation. Negative and positive control reactions were prepared. After that, the electrophoretic separation of 5µl of each resulting PCR product was used.

2.3.3 Sequencing Library preparation of human and mice samples

Illumina indices and adapters were added to amplicon libraries for multiplexing utilising the Nextera DNA Library Kit (Illumina, FC-121-1031) protocol as per manufacturer's instructions. The index adapter sequences on both ends of the amplicon were added by the PCR step, which facilitates double-index sequencing of clustered libraries on the Illumina sequencing schemes.

The following amplicons were normalised utilising the Agilent Bioanalyzer and clustered for sequencing at a concentration of 2nM. To generate the needed complexity for sequencing, 20% PhiX was added to the amplicons for a final amplicon's concentration of 4 pM.

2.3.3.1 Illumina MiSeq Sequencing

Barcoded 16S and ITS2 amplicons were sequenced utilising the Illumina MiSeq sequencing system with a 250bp paired-end read metric.

2.3.4 Bioinformatics of human and mice samples

Before utilising the Nephele 16S / ITS paired-end QIIME pipeline, the fastq files were pre-processed as follows; the Illumina adapter sequences were trimmed from the reads using Cutadapt version 1.2.1. The reads are further trimmed using Sickle version 1.200 with a minimum window quality score of 20 by sequencing company (Centre for Genomic Research -University of Liverpool, UK). Bioinformatic analysis was carried out utilising QIIME applied as a part of the Nephele 16S / ITS paired-end QIIME pipeline utilising open reference clustering against the SILVA database for bacteria and the ITS database for fungi at a sequence detect of 99%. Steps for data processing using QIIME for data obtained have been seen in **Table-2.6**.

QIIME 16S rRNA and ITS2 pipeline parameters of processing step were Phred Quality Scores =19 (A Phred quality score is a measure of the quality of the identification of the

nucleobases produced by automated DNA sequencing such as Illumina. Minimum Phred quality score of 19 for Q20 (1 in 100 of incorrect base call) or better is recommended. The default is 19), Phred offset =33 (Phred Q scores are often represented as ASCII characters of base 33 and 64. Base 33 is the most common representation on modern sequencing platforms, while 64 on 454 and older Illumina. The default is base 33), and the maximum Ambiguous = 0 (Maximum number of produce bases (N) allowed in a sequence to retain it. This is applied after quality trimming and is total over combined paired-end reads if applicable. The default is 0. For join reads step, max bad run length=3 (Maximum number of consecutive low-quality base calls permitted before truncation, the default is 3), minimum overlap=10 (Minimum number of overlapped bases for the join of paired-end reads, the default is 10), and percent difference within overlap =25 (Maximum percentage of differences in the overlapped regions, the default is 25%) (Caporaso *et al.*, 2010).

Table 2-6 Steps for data processing using QIIME (Caporaso *et al.*, 2010)

1-	Join forward and reverse short reads as contigs
2-	Dereplicate contig sequences
3-	Taxonomic assignment based on a selected database
4-	Identify and remove chimeric sequences
5-	Remove rare OTUs in the samples
6-	Detect differentially abundant features in samples
7-	Construct a phylogenetic tree
8-	Calculate various measures of diversity

2.3.5 **Statistical analysis of microbiome population in biological samples of humans and mice**

The statistical significance of differences in the abundance of human blood individual microbiome among the RA, AS, PA, and control donors were determined by the Kruskal-Wallis test, with correction for multiple testing via the original FDR method of Benjamini and Hochberg method. To identify differentially abundant bacterial species between pre- and post-treatment RA patients (RA V0 and RA V3), a Wilcoxon matched-pairs signed-rank test was used. Further, the statistical significance of differences in the abundance of bacteria and fungi taxa between the RA synovial fluid and control healthy subjects was determined by a Mann–Whitney U test, following in all cases, $P \leq 0.05$ was considered statistically significant. Furthermore, the statistical significance of differences in the abundance of taxa in CIA mice relative to control was determined by a Mann–Whitney U test. It should be noted that all statistical methods were used for each taxon expressed as a percentage of the total taxa on a per-sample basis. In all cases, $P \leq 0.05$ was considered statistically significant.

2.4 Cytokines detection

2.4.1 Human Magnetic Luminex Screening Assay

Serum, plasma, and synovial fluid cytokines were measured utilising a Human Magnetic Luminex Screening Assay following the manufacturer's instructions (R&D Systems, Minneapolis, USA). Levels of IL-6, IL-17, IL-22, and IL-23 cytokines were analysed by the LXSAHM-04 kit. This work was done by labospace (Milano, Italy). Briefly, serum, plasma, and synovial fluid samples were centrifuged at 16,000 x g for four minutes. Prior to analysing, the serum, plasma, and synovial fluid samples were diluted in 1:2 ratios by adding 25 µl of the serum, plasma, and synovial fluid samples and 25 µl of Assay Buffer. The biofluids samples and standards were added to the wells Assay. After that, 50 µl of diluted Microparticle Cocktail was added to each well, and the plate assay was then closed and incubated at room temperature for 2 hours on a plate shaker (800 rpm). The following wells were washed by 100 µl Wash Buffer 3 times, and 50 µl of diluted Biotin-Antibody Cocktail was added to each well, and the plate was sealed and incubated at room temperature for 1 hour on a plate shaker (400 rpm). Again, the sample was washed by 100ul Wash Buffer. After this, 50 µl of diluted Streptavidin-PE was added to each well and incubated for 30 minutes at room temperature on the plate shaker at 800 rpm. Next, the washing step with100ul Wash Buffer was repeated twice to ensure removing all the liquid from each well and incubated for two minutes at room temperature on the plate shaker at 800 rpm. Within 90 minutes, the samples were read using Bio-Rad analyser.

2.4.2 **Statistical analysis**

The statistical significance of differences in cytokines levels of serum, and plasma samples among the RA, AS, PA, and control donors were determined by the Kruskal-Wallis test, with correction for multiple testing via the original FDR method of Benjamini and Hochberg method. Further, the statistical significance of differences in the cytokine's levels of synovial fluid samples between the RA synovial fluid and control donors was determined by unpaired T-test, following in all cases, $P \leq 0.05$ was considered statistically significant. The correlation between microbiome taxa and cytokine profiles concentrations were analysed via Spearman's correlation using GraphPad 8 software.

Chapter 3

3 Characterisation of the blood microbiome of patients with rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, and healthy control subjects

3.1 Overview

Mounting evidence propose that the composition and status of the blood microbiome may play a critical role in the pathogenesis of various diseases states (diabetes, cardiovascular disease, atherosclerosis, and in Kawasaki disease) (Amar *et al.*, 2011, 2013; Vajro, Paolella and Fasano, 2013; Trøseid *et al.*, 2014; Abe *et al.*, 2015). These investigations pose various questions around the nature of their presence and their function in health and illness. Furthermore, it supports the concept that the microbiome can circulate in the blood circulation of well-appearing humans, even if transiently. Despite mounting evidence suggesting a pivotal role for dysbiosis in the initiation and development of RA, AS, PA illnesses, no study to date has investigated whether such translocation occurs in these highly prevalent disorders. I believe that these nucleic acids have leached from classical microbiome niches into the blood and may represent novel biomarkers for disease pathogenesis, hence the novelty of the study.

This chapter aims to characterise the blood microbiome of patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS), and psoriatic arthritis (PA), relative to healthy control subjects. This will help to identify specific microbiome signatures associated with RA, AS, and PA illnesses, which may increase our understanding of pathogenesis and or reveal a pool of candidate biomarkers for further development. Hence, here, I also determine the correlation of inflammatory cytokines (IL-17-A, IL-22, IL23, and IL-6) with these inflammatory conditions.

3.2 Methods

Methods for 16S rRNA and ITS2 PCR steps, Gel electrophoresis, DNA purification by Qiagen Purification Kit, Addition of Illumina XT tags, DNA purification by AMPure XP magnetic beads, DNA sequencing utilising an Illumina MiSeq and subsequent bioinformatic analysis, and further, detection of the IL-6, IL-17A, IL22, and IL-23 levels in the blood are detailed in the **Methods chapter.**

3.3 Results

3.3.1 Donor Population

This study investigated the presence of bacterial and fungal DNA in a range of donated blood samples. A total of forty-four subjects were included in the study. Among these, twenty were obtained from Haywood hospital (Staffordshire, UK). All of them were diagnosed with rheumatoid arthritis and provided serum samples on their first visit (RA V0) and following (RA V3) the instigation of three months of treatment. In addition to the RA patient serum, four plasma samples from patients with RA, four serum samples from ankylosing spondylitis (AS) patients, four serum and four plasma samples from psoriatic arthritis (PA) patients, and four serum and four plasma samples were obtained from healthy volunteers who had no clinical symptoms of RA and others diseases which were obtained to allow comparison among the various disease states. All these samples were obtained from the Sera Laboratories Limited, UK.

3.3.2 **16S rRNA PCR amplification**

We normalised the volume of serum and plasma at the point of PCR to 4ul from each sample. Normalisation step help to obtain consistent, reliable data when working with multiple samples. Following PCR as described in **2.3.1.1**, bacterial 16S rRNA was detected in 90% of RA serum patients, which provided a total of seventeen paired samples for analysis **(i.e., those subjects for which V0 and V3 data was available)**. In addition to the RA patient serum, bacterial 16S rRNA was found in 50% (2/4) of RA plasma samples, 100% (4/4) of AS serum samples, 50% (2/4 of serum and 2/4 of plasma samples) PA patients, and 50 % (4/4 of serum, and 0/4 of plasma samples) healthy subjects providing a total of fourteen samples for analysis. Our various experimental negative controls (the negative controls of PCR experiments/ kit controls of purification step by PCR Purification Kit) constantly failed to generate a visible band after PCR and agarose gel electrophoresis. In addition, DNA quantification utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen) confirmed the absence of DNA from the negative controls. The Qubit 3.0 high-sensitivity DNA kit is designed to be accurate for initial sample concentrations from 10 pg/µL; in the case of our negative control samples, zero values were obtained. As an additional precautionary measure, I reviewed the additional negative control reactions that were sequenced on the same run and at the same time as the samples mentioned here. One such sample produced mappable sequencing data (sample NEGF) and contained a small number of reads mapping to the genera *Halomonas* (6 reads), *Corynebacterium 1* (64), *Staphylococcus* (24), *Ralstonia* (1726), *Stenotrophomonas* (460), *Pseudomonas* (276), and *Ruminococcus* (405), but predominately contained reads mapping to the genera Escherichia-*Shigella** (2420), and *Serratia** (18000). This was from another study but produced using the identical methodology to that used herein.

Many of these taxa have been identified previously as contaminants of next-generation sequencing experiments (Laurence, Hatzis and Brash, 2014; Salter *et al.*, 2014a) but a significant point herein, were either distinct from the taxa found within our samples or existent at much lower levels. To control for this possible source of contamination, we highlight any taxa that were found within sample NEGF at a level > 25% of the mean experimental sample level for example, on average, we require a likely contaminant to be seen at a level higher alternatively, equal to four times that detected in the negative control to recognise its presence as reliable. The utilisation of this method found *Serratia, Escherichia-Shigella, Ralstonia* and *Ruminococcus* as possible contaminants and discussion of these taxa will make reference to this fact.

3.3.3 **Characterisation of the circulating bacterial community via 16S rRNA sequencing**

The presence of microbial DNA in the human blood samples (serum and plasma) was assessed by way of PCR amplification and sequencing of the bacterial 16S rDNA gene, followed by bioinformatic analysis utilising QIIME **(as shown in methods chapter, 2.3.4)**. Our first approach used Principal coordinates analysis (PCoA) to decrease the complexity of the data obtained and to visualise any apparent alterations in clustering among samples of different diseases types **(Figure 3-1).** Following ordination, it is noted that the clusters of RA serum samples separately to those from other biological samples (RA plasma, AS serum, serum and plasma of PA, and control serum samples). The results of the PCoA plots also point out that the types of microbiome present in the Sera Laboratories and

Haywood hospital samples are very different. There is also a wide spread of microbiome variation across the RA samples.

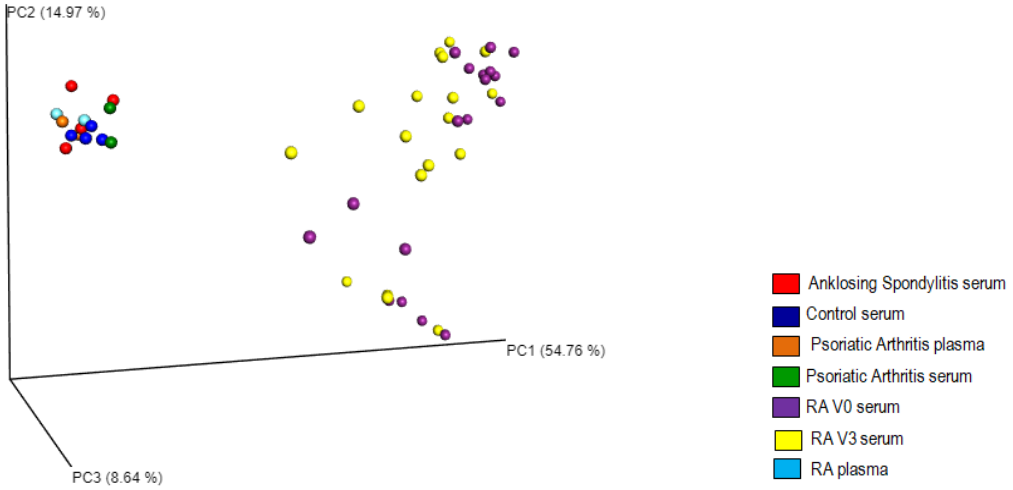


Figure 3-1 PCoA plot produced by the use of weighted unifrac distance matrix of 16S rRNA for the serum of RA V0 (purple), and RA V3 (yellow) (RA V0 and RA V3 are taken from Haywood hospital samples), RA plasma (sky blue), AS serum (red), PA serum (green), PA plasma (orange) and control subjects (blue) (RA plasma, AS serum, PA serum and plasma, and control subjects serum from are obtained the Sera Laboratories) as determined through amplification and sequencing of 16S rRNA, variable region 4. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA identified that the maximal variation was 54.76% (PC1), 14.97 % (PC2) and, 8.64% (PC3). The microbiota of samples that appear in close proximity to each other is considered to have more similar microbiome communities. Following ordination, it is fascinating to note that the clusters of RA serum samples separately to those from other biological samples (RA plasma, AS serum, serum and plasma of PA, and control serum samples). The results of the PCoA plots also show that the types of microbiome present in the Sera Laboratories and Haywood hospital samples are very different. There is also a wide spread of microbiome variation across the RA serum samples.

3.3.4 Serum microbiome composition according to treatment response and seropositivity

Principal Coordinate 1 represents > 50% of the total variation in the data set. Samples appeared to be clustering along this axis (X) with control samples clustered to the right, and RA samples clustered to the left. My supervisor Dr. Dan Tonge made the PCoA approach. Due to the paired nature of our RA cohort, we linked pre and post-treatment measurements within each patient (V0 and V3 revealed in pink and blue, respectively) via means of a black connecting line. For 13/17 pairs of samples, the microbial population observed to progress along Principal Coordinate 1 after treatment, proposing that the blood microbiome population in these samples turned to more similar to that of the healthy control / PA/AS cohort after treatment.

In order to examine this effect in more detail, we have seen the above coordination in light of the type of treatment **(Figure 3-2)** and RF/CCP status **(Figure 3-3).** Of the 17 RA patients who had complete information associated with successful microbial analysis before and after treatment, six patients were prescribed methotrexate monotherapy (MTX), two patients' methotrexate and hydroxychloroquine (MTX_HCQ), one patient methotrexate and sulphasalazine (MTX_SSZ), and eight patients received a mixture of all three drugs (MTX_HCQ_SSZ) (**See Table-2.1 in methods chapter)**. Thirteen pairs of a sample that progressed toward the right along PC1 post-treatment came from patients treated with a variety of medications (MTX, MTX_HCQ, MTX_HCQ_SSZ, and MTX_SSZ). In addition, the four patients who did not progress in this direction also came from patients treated with a range of approaches (MTX, MTX_HCQ, MTX_HCQ_SSZ, and MTX_SSZ), proposing that the observed change in microbiome population was not affected by the treatment method. Conversely, of the 13 patients whom that progressed toward the right along with

PC1 post-treatment, ten (77%) patients had negative RF / CCP, and only three patients had positive RF / CCP. In contrast, 3 out of the 4 (75%) patients that did not progress towards the right post-treatment had RF/CCP positive. These data propose that the role of RF / CCP in modulating the microbiome response after the onset of treatment with those patients with RF / CCP negative RA more likely to progress toward a control/ PA/AS microbiome population than those who consider RF / CCP to be positive.

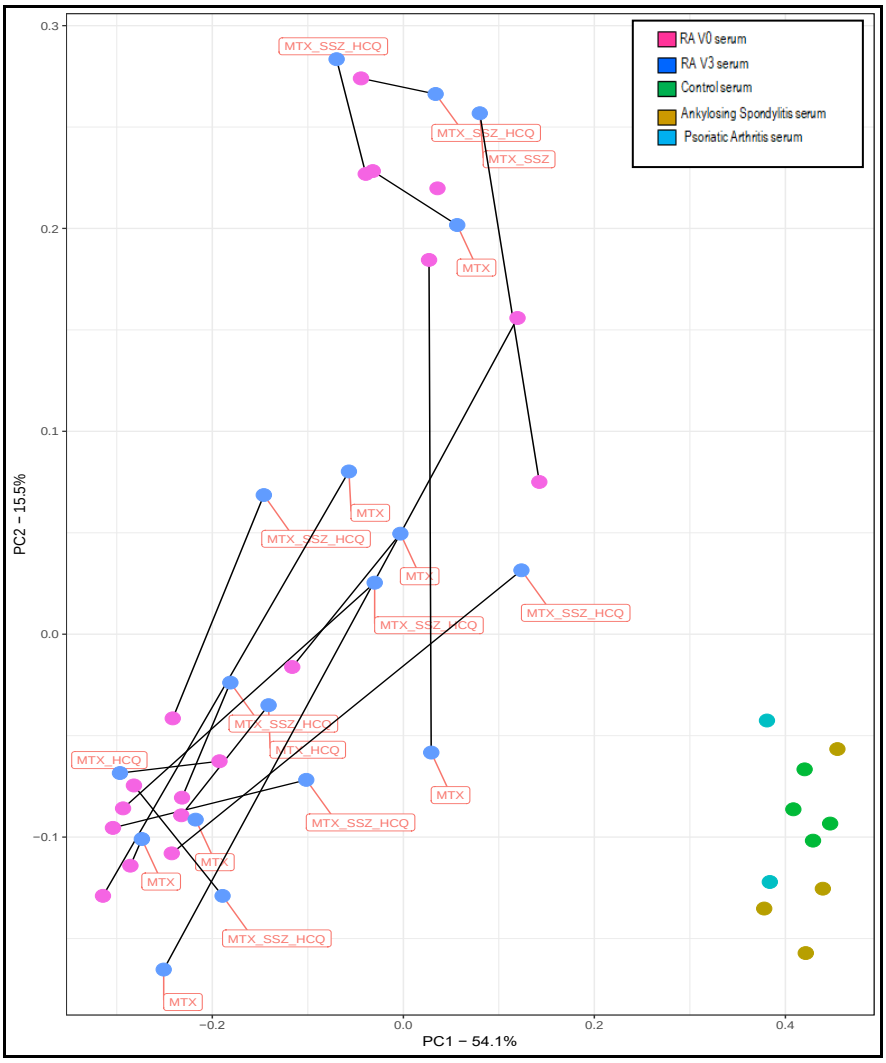


Figure 3-2 PCoA plot produced by the use of weighted unifrac distance matrix for RA V0 (pink), and RA V3 (blue), AS (mustard), PA (aqua), and control subjects (green). Distance matrix informed by amplification and sequencing of the 16S rRNA variable region 4, followed by the taxonomic assignment, whereas samples are labelled by treatment type. Where applicable, paired-samples (V0, V3) are linked through a black line. Proportions of variation explained by principal coordinates 1 and 2 are designated on the relevant axes. Variation explained by the PCoA axes of ordination was 54.1% (PC1) and 15.5 % (PC2).

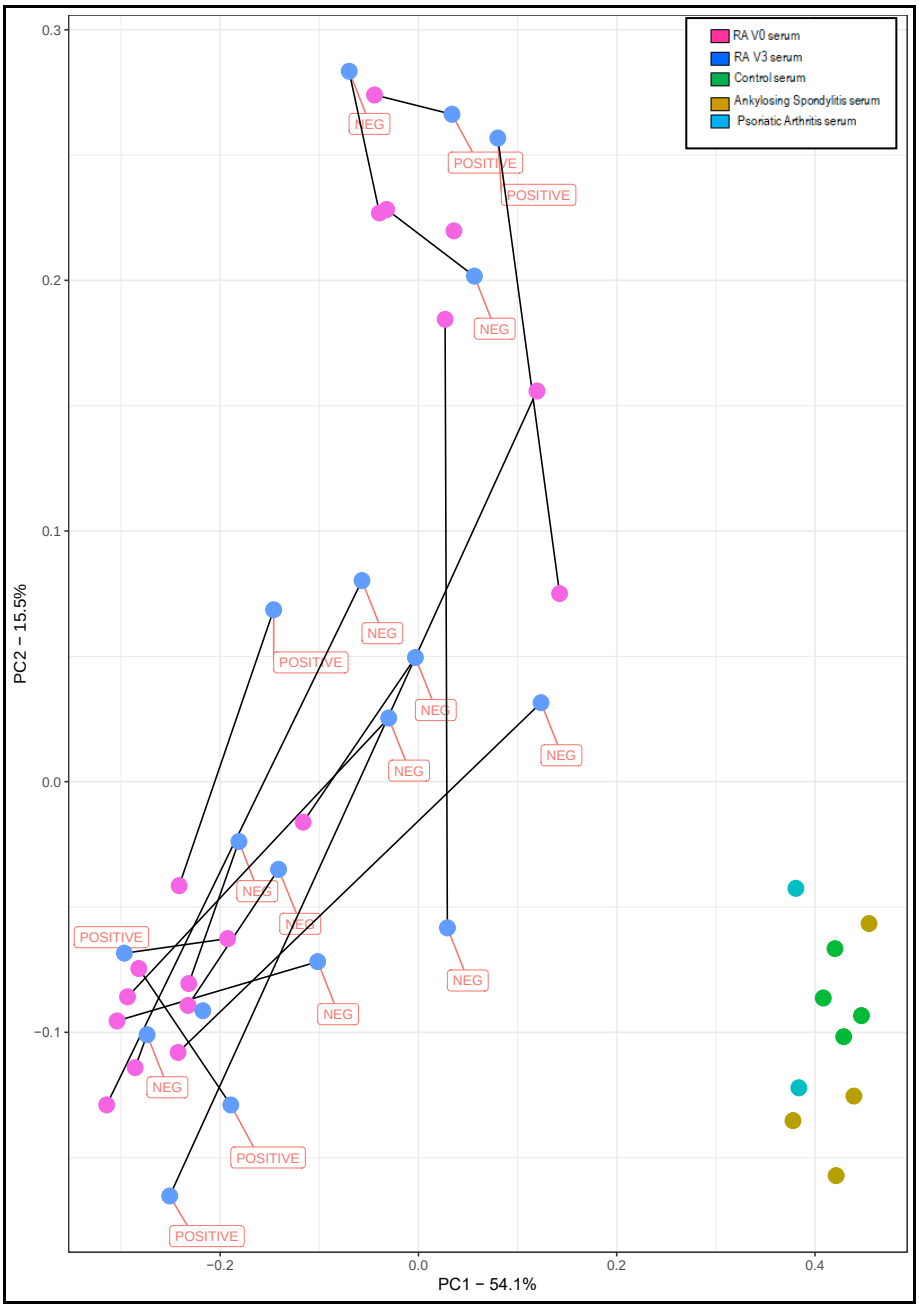


Figure 3-3 PCoA plot produced by the use of weighted unifrac distance matrix for RA V0 (pink), and RA V3 (blue), PA (aqua), AS (mustard), and control subjects (green). Distance matrix informed by amplification and sequencing of the 16S rRNA variable region 4 followed by the taxonomic assignment, whereas samples are labelled by RF/CCP status. Where applicable, paired-samples (V0, V3) are linked through a black line. Proportions of variation explained by principal coordinates 1 and 2 are designated on the relevant axes. Variation explained by the PCoA axes of ordination was 54.1% (PC1) and 15.5 % (PC2).

3.3.5 **Bacterial Community Composition**

We do have a measure of total abundance and have absolute abundance data for every taxon - this is read count for each taxon. These are routinely considered in addition to the following; to compare samples with different sequencing depths, each taxon was expressed as a percentage of the total number of reads for that given sample.

It should be noted that we standardised the amount of biofluid used in the PCR reaction, and library normalization prior to sequencing (this standardizes each library in an equimolar concentration to ensure roughly the same sequencing depth per sample. Also, it is ensured that the sequencing depth was not limiting by undertaking a rarefaction analysis. This is standard practice and well-published.

Another next-generation sequencing such as miRNA-seq also their genes expression account are expressed as a percentage of the total number of reads for that given sample.

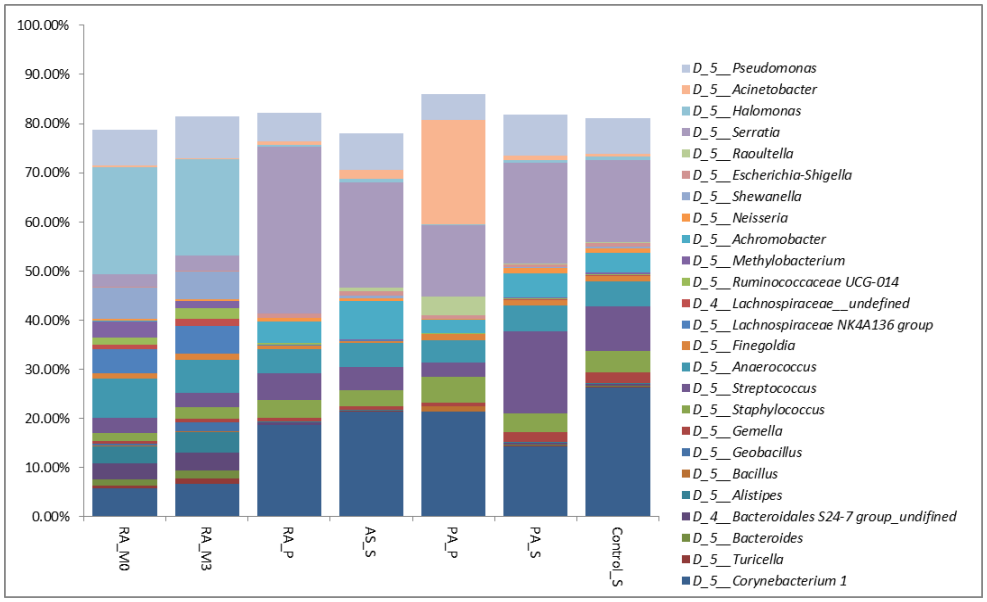
At the phylum level, our blood samples were dominated by *Proteobacteria* (46.1% of all bacterial DNA), followed by *Firmicutes (30.8%), Actinobacteria (10.9%), and Bacteroidetes (10.5%).* These results mirror previous studies (Amar *et al.*, 2013; Païssé *et al.*, 2016; Olde Loohuis *et al.*, 2018; Whittle *et al.*, 2019) and further support the notion of a core blood microbiome predominated by four key phyla.

At the genus level, our RA serum samples were predominated by genera *Halomonas* (20.6%), *Anaerococcus* (7.4%), *Pseudomonas* (7.85%), *Corynebacterium 1* (6.15%), *Shewanella* (6.1%) and *Lachnospiraceae NK4A136* group (5.15%), as shown in **Figure 3-4.** In contrast, control serum samples were predominated by the genera *Corynebacterium* (26.3%), *Serratia** (17.10%), *Streptococcus* (9.1%), *Pseudomonas* (7.3%), *Anaerococcus* (5.0%), *Staphylococcu*s (4.3%) and *Achromobacter* (4.0%). Many of these genera were

identified in the blood of healthy human donors as part of an entirely separate study (Whittle et al., 2018), albeit in differing proportions. Moreover, RA plasma samples were comprised genera *Serratia** (34%), *Corynebacterium 1* (18.6%), *Pseudomonas* (5.8%), *Streptococcus* (5.4%), *Anaerococcus (4.9%), Achromobacter* (4.3%) and *Staphylococcus* (3.6%). Furthermore, serum samples from AS patients contained the same core genera of control serum samples and were dominated by genera Serratia* (21.6%), *Corynebacterium 1* (26.3%), Achromobacter (7.8%), *Pseudomonas* (7.5%), *Anaerococcus* (5.0%), *Streptococcus* (4.7%), *and Staphylococcus* (3.3%). Circulating samples from our PA were comprised genera *Corynebacterium 1* (17.75%), *Serratia** (17.5%), *Streptococcus (*9.85%), *Pseudomonas* (7.2%), *Anaerococcus* (4.85%), *Achromobacter* (3.5%) and *Staphylococcus* (4.55%).

*Potential contaminant present within a single negative control reaction at a level exceeding that showed in our experimental samples.

A-



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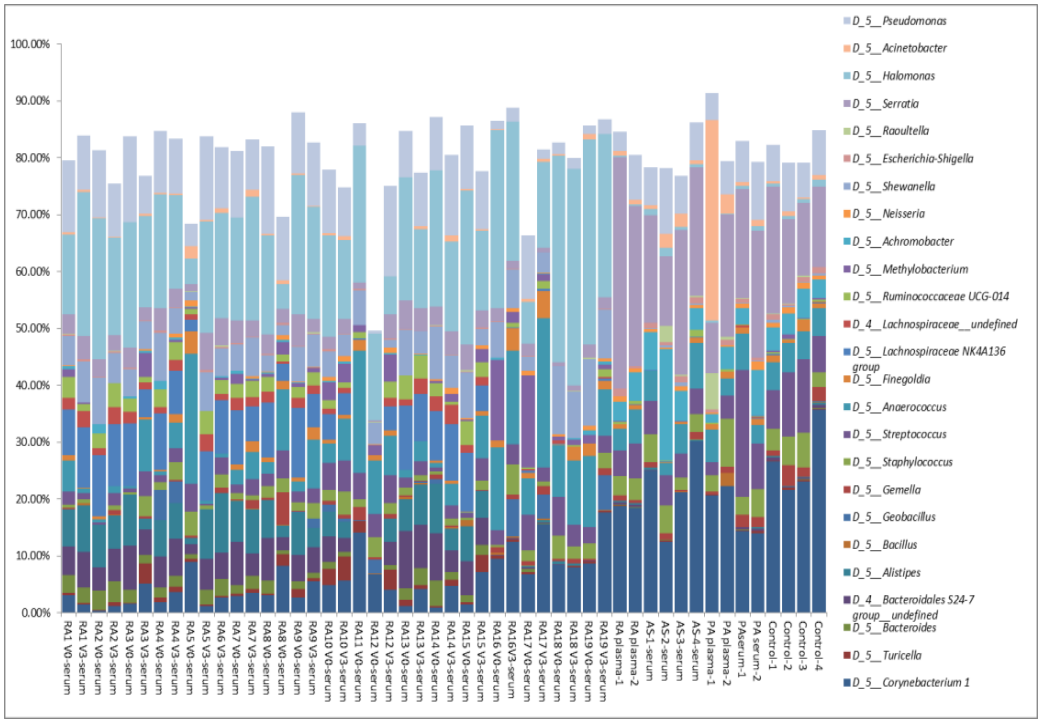


Figure 3-4 Relative abundance of the most abundant bacterial genera. Data are the relative abundance of the major bacterial taxa, characterized as having a mean abundance of >1%, in the serum of rheumatoid arthritis (RA V0, n = 18, and RA V3, n = 18), RA plasma, n=2, serum samples from ankylosing spondylitis (AS, n = 4), serum and plasma from psoriatic arthritis (PA serum, n =2, and PA plasma, n=2), and Control serum (Control, n = 4) samples as determined using amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean abundance expressed as a percentage of the total bacterial sequence count. (A) Taxa data grouped by the condition of bacteria in the blood, and (B) Taxa individual sample data of bacteria in the blood.

Statistical analysis of those genera which represent at least 1% of any one experimental group was conducted utilising the Kruskal Wallis test. Seventeen taxa were significantly changed via illness status and are observed in **Table 3-1.** Post hoc analysis was carried out utilising the Benjamini-Hochberg correction for multiple comparisons in Graphpad prism version 8.

Table 3-1 Taxa significantly changed via illness status. In this table, we compared the bacterial taxa in all diseased conditions (RA, AS, and PA) against healthy control subjects. Statistical analysis was conducted utilising the Kruskal Wallis test, followed by the Benjamini-Hochberg correction for multiple comparisons to identify the microbiome significantly shifted via the diseased condition

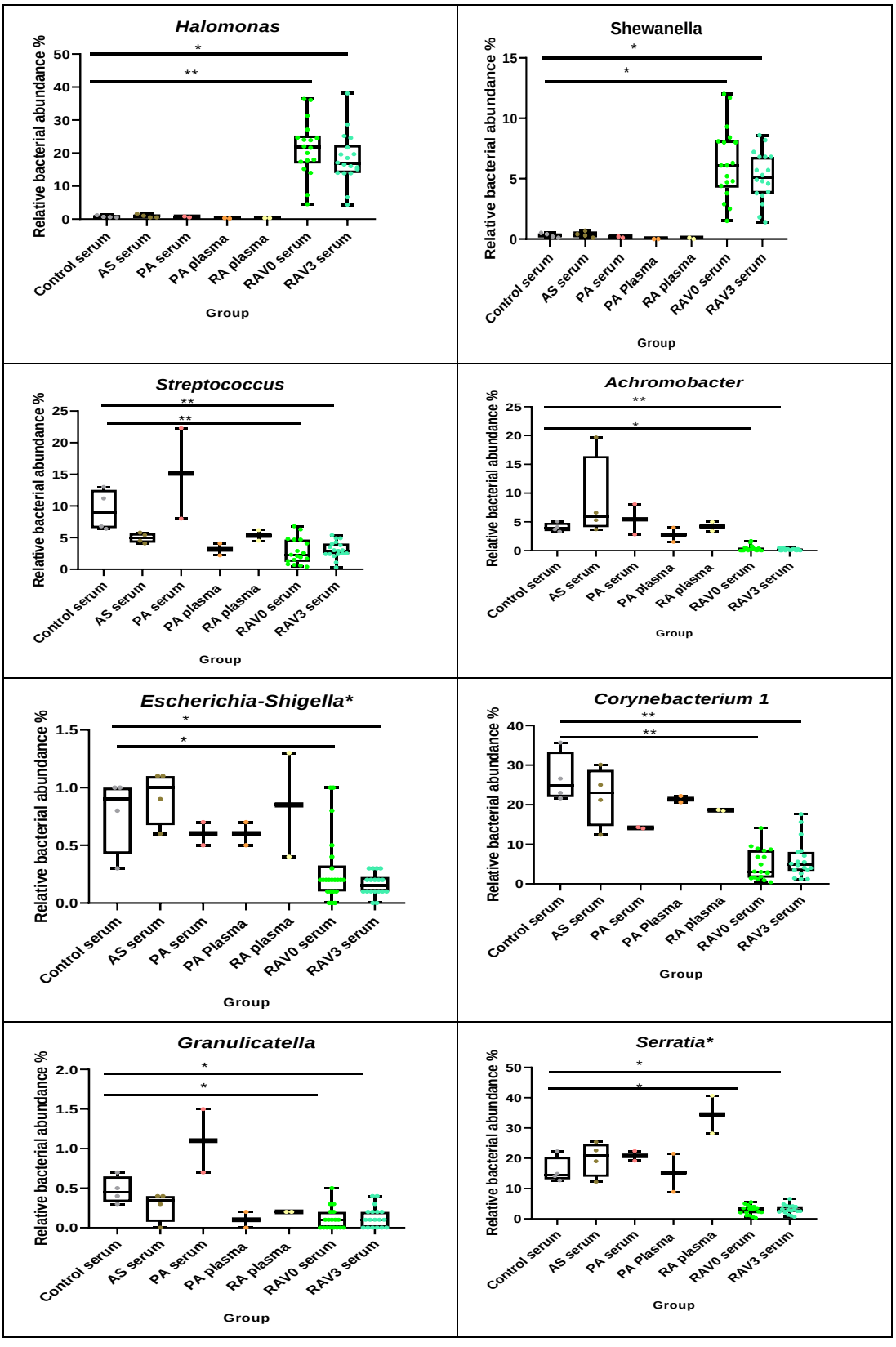
N	Taxonomy	Kruskal Wallis	PA serum FDR	PA plasma FDR	AS serum FDR	RA plasma FDR	RA V0 serum FDR	RA V3 serum FDR
		P value	P value	P value	P value	P value	P value	P value
1	*D_5__Halomonas*	<0.0001	ns	ns	ns	ns	0.009	0.034
2	*D_5__Shewanella*	<0.0001	ns	ns	ns	ns	0.013	0.026
3	*D_5__Achromobacter*	<0.0001	ns	ns	ns	ns	0.012	0.009
4	*D_5__Serratia**	<0.0001	ns	ns	ns	ns	0.016	0.016
5	*D_5__Corynebacterium 1*	<0.0001	ns	ns	ns	ns	0.001	0.003
6	*D_5__ Escherichia-Shigella**	0.0003	ns	ns	ns	ns	0.039	0.015
7	*D_5__Streptococcus*	0.001	ns	ns	ns	ns	0.003	0.006
8	*D_5__Chryseobacterium*	0.003	ns	ns	ns	ns	ns	ns
9	*D_5__Staphylococcus*	0.005	ns	ns	ns	ns	0.02	ns
10	*D_5__Granulicatella*	0.007	ns	ns	ns	ns	0.01	0.02
11	*D_5__Gemella*	0.009	ns	ns	ns	ns	0.006	0.04
12	*D_5__Acinetobacter*	0.01	ns	ns	ns	ns	ns	ns
13	*D_5__Ruminococcaceae UCG-014*	0.01	ns	ns	ns	ns	ns	ns
14	*D_5__Turicella*	0.013	ns	ns	ns	ns	ns	ns
15	*D_5__Neisseria*	0.019	ns	ns	ns	ns	ns	ns
16	*D_5__Alistipes*	0.02	ns	ns	ns	ns	ns	ns
17	*D_5__Methylobacterium*	0.042	ns	ns	ns	ns	ns	ns

* Taxa previously associated with contamination and identified within a single negative control reaction. ns, not statistically significant, FDR, False Discovery Rates.

Statistical analysis identified that the abundance of the genus of *Halomonas,* and *Shewanella,* were significantly increased in the serum of RA patients (RA V0 and RAV3) in comparison to the healthy donor's serum. In contrast, serum RA patients (RA V0 and RAV3) have significantly decreased in the abundance of *genus Achromobacter, Escherichia-Shigella*, Serratia*, Corynebacterium 1, Streptococcus, Granulicatella,* and *Gemella* relative to control subjects. Further, the abundance of genus *Staphylococcus* was decreased in naïve RA patients (RA V0) in comparison to control subjects **(Figure 3-5, and Table 3-2).**

Table 3-2 Median relative abundance (SD) comparison between diseased and control subjects.

Bacterial Genera	Control-S Median (SD)	AS-S Median (SD)	PA -S Median (SD)	PA-P Median (SD)	RA-P Median (SD)	RAV0-S Median (SD)	RAV3-S Median (SD)
Halomonas	0.65(0.31)	0.75(0.52)	0.65(0.21)	0.25(0.07)	0.3(0)	21.8(8.5)	16.8(7.7)
Shewanella	0.3(0.18)	0.35(0.25)	0.15(0.07)	0(0)	0.05(0.07)	6.1(3)	5.1(2)
Streptococcus	9 (3.2)	5(0.7)	15.1(10.1)	3.1(1.3)	5.3(1.2)	2.2(1.9)	3(1.3)
Achromobacter	3.9(0.7)	6(7.3)	5.4(3.7)	2.7(1.7)	4.2(1.1)	0.2(0.5)	0.1(0.17)
Escherichia-Shigella*	0.9(0.3)	1(0.2)	0.6(0.14)	0.6(0.14)	0.85(0.6)	0.2 (0.3)	0.15(0.1)
Corynebacterium 1	24.8 (6.3)	23.1(7.4)	14.1(0.2)	21.4(1.1)	18.6(0.1.4)	3(3.8)	4.9(4.7)
Granulicatella	0.45(0.17)	0.35(0.18)	1.1(0.5)	0.1(0.14)	0.2(0)	0.1(0.14)	0.1 (0.13)
Serratia*	14.5(4.3)	20.8(5.7)	20.8(2.3)	15.2(8.9)	34.4(8.8)	3.2(1.4)	2.8(1.4)
Gemella	2 (1.2)	1(0.4)	1.9(0.2)	0.8(0.4)	0.8(0.3)	0.3(0.5)	0.7(1.3)
Staphylococcus	4.4(1.5)	3.9(1.3)	4(1.2)	5.7(3.9)	3.6(0.3)	1.3(1.3)	2 (1.3)


Halomonas
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Shewanella
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA Plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Streptococcus
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Achromobacter
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Escherichia-Shigella*
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA Plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Corynebacterium 1
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA Plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Granulicatella
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA plasma
RA plasma
RAV0 serum
RAV3 serum
Group
Serratia*
Relative bacterial abundance %
Control serum
AS serum
PA serum
PA Plasma
RA plasma
RAV0 serum
RAV3 serum
Group

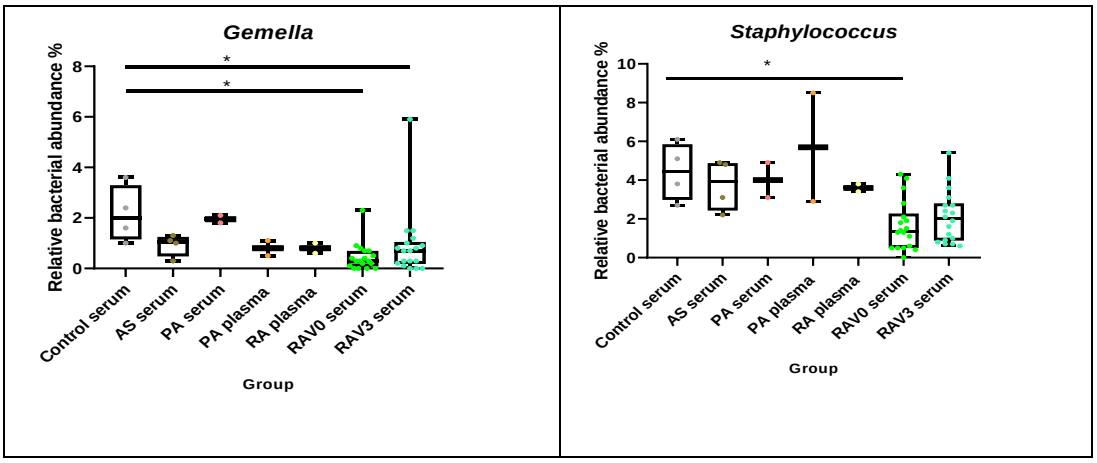


Figure 3-5 Relative abundance of *Halomonas, Shewanella, Streptococcus, Achromobacter, Escherichia-Shigella*, Corynebacterium 1, Serratia*, Granulicatella, Gemella, and Staphylococcus* detected in the serum of rheumatoid arthritis (RA V0 and RA V3), plasma RA, serum AS, serum and plasma PA and healthy control serum. Data determined through the amplification and sequencing of the 16S rRNA gene. The box and whiskers are showing the distribution of the data. The original FDR method of Benjamini and Hochberg showed the RA serum (RA V0 and RA V3) was associated with significantly increased of the abundance of the genus *Halomoans*, and *Shewanella* increased significantly decreased the abundance of genera *Streptococcus, Achromobacter, Escherichia-Shigella*, Corynebacterium 1, Serratia*, Granulicatella, and Gemella* relative to our healthy control subjects. Further, there was a significant decrease in genus *staphylococcus* in RA V0 relative to healthy control subjects. Data are median abundance expressed as a percentage of the total bacterial sequence count. *P < 0.05; **P < 0.01.

To further explore the results of our ordination, which suggested that the circulating microbiome of many of our RA patients moved closer towards the healthy control state post-treatment, I analysed the V0 and V3 data for all taxa, considering the paired nature of these observations. Wilcoxon Signed-Rank Test analysis revealed that members of the genera *Haemophilus*, *Alloprevotella*, *Eremococcus,* and *Lachnospiraceae*_UCG001 were significantly altered between V0 and V3 **(Figure 3-6, and Table 3-3).**

Table 3-3 Median relative abundance (SD) comparison between RAV0 and RAV3 subjects and also compared with control subjects

Bacterial Genera	Control-S Mean(SD)	RAV0-S Mean(SD)	RAV3-S Mean(SD)
Haemophilus	0.75(0.31)	0.3(0)	21.4(8.5)
Alloprevotella	0.3(0.18)	0.05(0.07)	6.3(3)
Eremococcus	9.3(3.2)	5.3(1.2)	2.7(1.9)
Lachnospiraceae_UCG001	4(0.7)	4.2(1.3)	0.37(0.5)

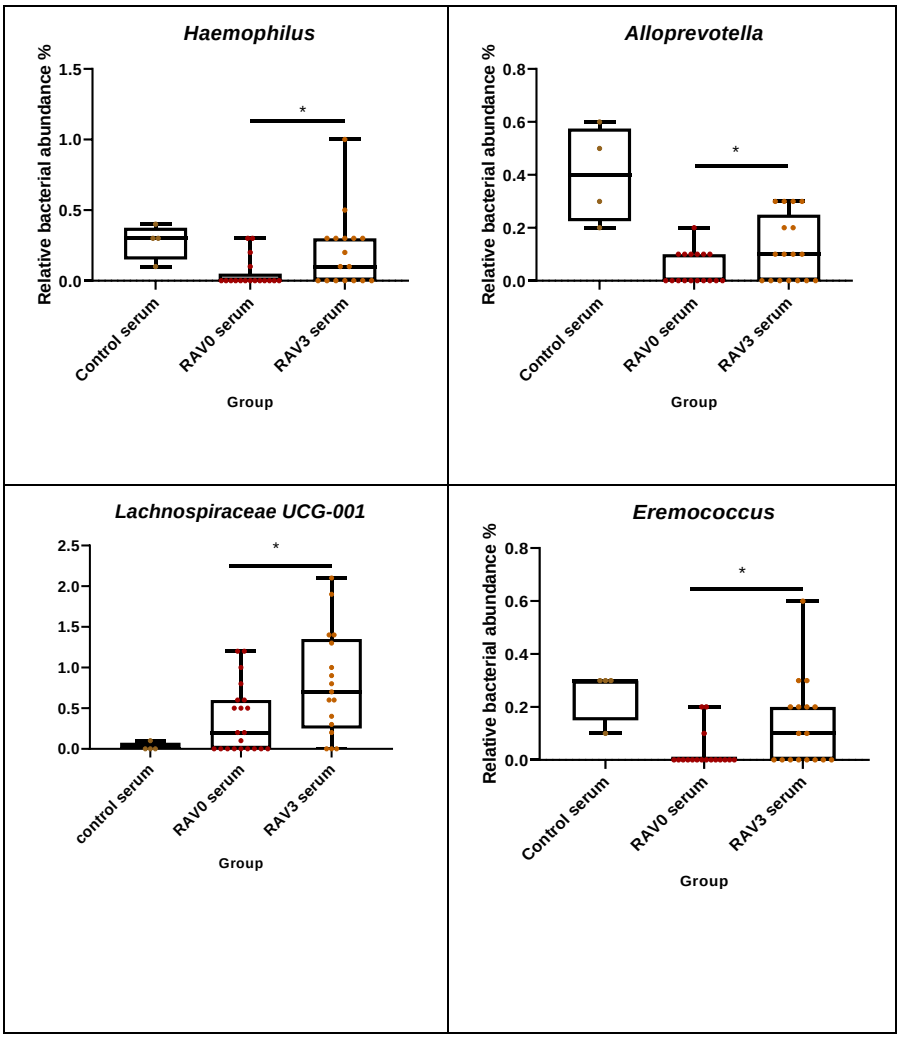


Figure 3-6 Relative abundance of *Haemophilus, Alloprevotella, Eremococcus,* and *Lachnospiraceae_UGC-001* identified in the serum of rheumatoid arthritis patients (RA V0 and RA V3). Data determined by the amplification and sequencing of the 16S rRNA gene variable region. The abundance of *Haemophilus, Alloprevotella, Eremococcus*, and *Lachnospiraceae_UGC-001* increased significantly in the serum of RA V3 relative to the serum of RA V0. Data are median abundance expressed as a percentage of the total bacterial sequence count. *P < 0.05.

3.3.6 **ITS2 PCR amplification**

Utilising PCR amplification, fungal ITS2 DNA was absent in the serum of RA patients (RA V0 and RA V3). However, ITS2 amplification, indicative of the presence of fungi, was detected in 1 of 4 (25%) plasma with RA, the serum of 3 of out 4 (75%) patients with AS, 2 out of 4 (50%) PA plasma, 3 out of 4 (75%) PA serum, 3 out of 4 (75%) healthy control subjects' serum, and 3 out of 4 (75%) in the plasma of healthy control. All experimental negative controls (the negative controls of PCR experiments/ kit controls of purification step by PCR Purification Kit) consistently failed to generate a visible band after PCR and agarose gel electrophoresis. Besides, DNA quantification utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen) confirmed this non-appearance.

3.3.7 **Characterisation of fungal populations via ITS2 sequencing of blood**

The existence of fungal DNA in the blood samples was assessed via way of PCR amplification and sequencing of the fungal ITS2 gene, followed by bioinformatic analysis using QIIME **(as shown in Methods Chapter, 2.3.4).**

Principal coordinates analysis (PCoA) was used to reduce the complexity of the data obtained, and to visualise any obvious clustering among samples of different illness types **(Figure 3-7).**

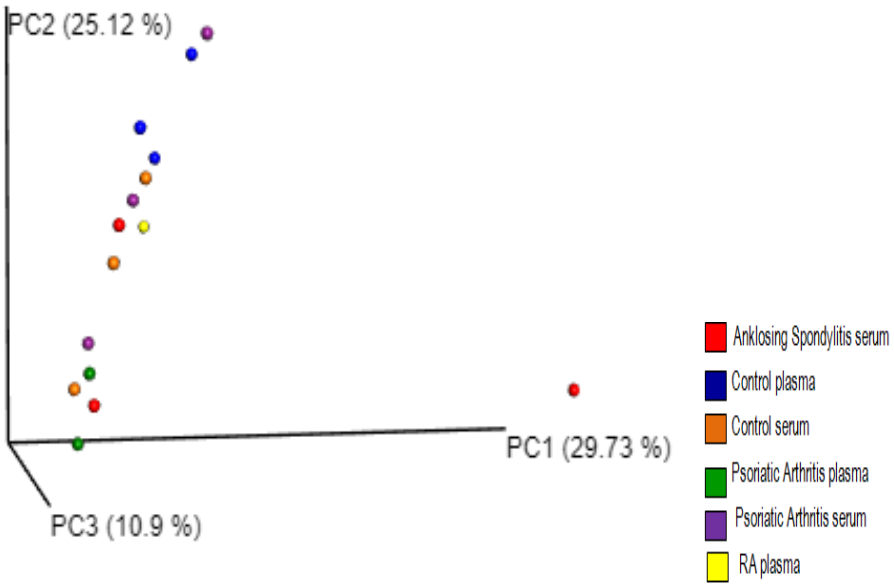


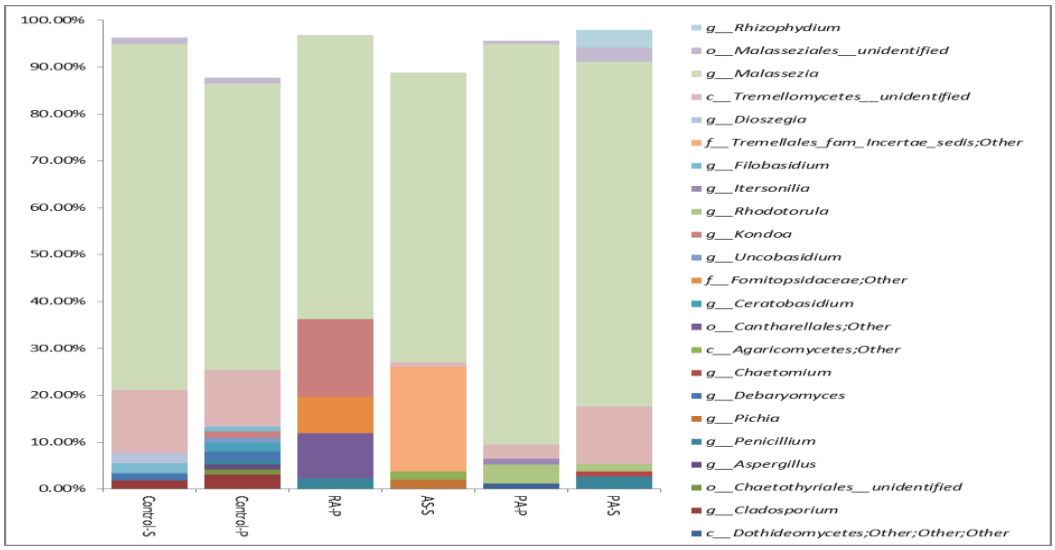
Figure 3-7 Principal Coordinate Analysis plot informed by a Bray Curtis distance matrix of blood fungal community structure for control subjects' serum (orange), control plasma (blue), arthritic patients' plasma (yellow), AS serum (red), PA plasma (green), PA serum (purple) as assessed via amplification and sequencing of the ITS2 gene. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA identified that the maximal variation was 29.73% (PC1), 25.12% (PC2) and, 10.9% (PC3). The microbiota of samples that appear in close proximity to each other is more similar in composition.

3.3.8 **Fungal Community Composition**

At the phylum level, blood was identified to be predominated by members of the *Basidiomycota* RA plasma= 94.5%, AS serum 86.9%%, PA plasma= 94.6%, PA serum= 90.2%, control serum= 93.5%, and control plasma= 80.4%) and *Ascomycota* (RA plasma= 2.3%, AS serum= 2.0%, PA plasma= 2.1%, PA serum= 3.8%, control serum=4.9%, and control plasma=14.9%) phyla.

At the genus level **(Figure 3-8),** our blood samples were dominated via by the genus *Malassezia* (RA plasma= 60.5%, AS serum= 61.9%, PA plasma= 85.4%, PA serum= 73.5%, control serum=73.8%, and control plasma= 61.2%), followed by specific unclassified organisms belonging to the classes Tremellomycetes (RA plasma= 0.0%, AS serum= 1.0%, PA plasma= 3.0%, PA serum= 12.2%, control serum=13.5%, and control plasma= 11.9%). To a lesser extent, the blood samples contained specific unclassified organisms belonging to the *Malasseziales* order (RA plasma= 0.0%, AS serum= 0.0%, PA plasma= 0.8%, PA serum= 3%, control serum=1.3%, and control plasma= 1.3%), and genus *Kondoa* (RA plasma= 16.6%, AS serum= 0.0%, PA plasma= 0%, PA serum= 0%, control serum=0%, and control plasma= 1.3%). Further, unclassified organisms belonging to the family *Tremellales_fam_Incertae_sedis* was identified only AS serum group (22.3%), and genus *Rhizophydium* (3.9%) detected in PA serum, however, genus *Cladosporium* identified in control group (control serum= 1.9% and control plasma= 3.1%) **.** The statistical analysis identified that the abundance of the fungal population was unaltered via illness condition.

A-



B-

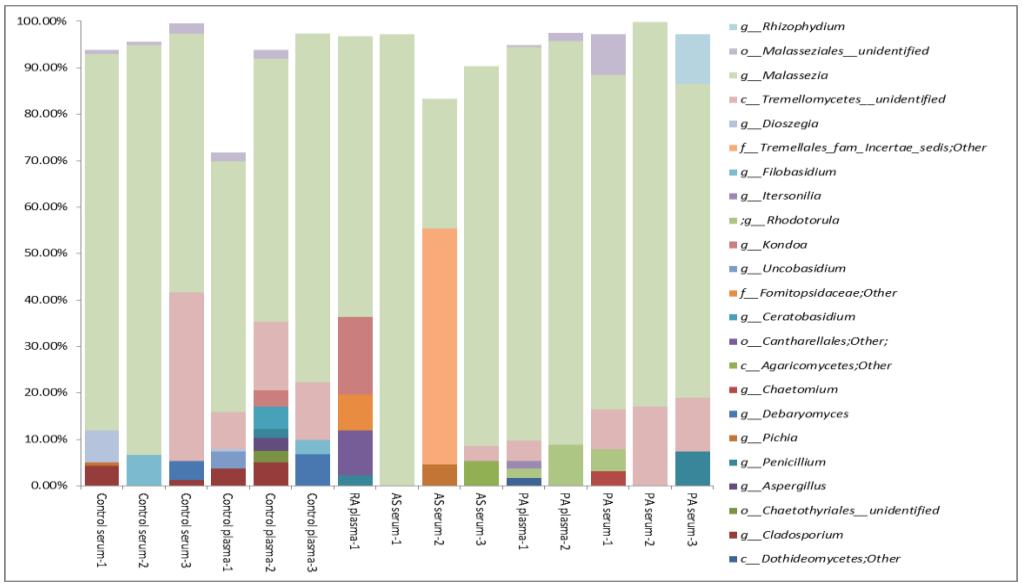


Figure 3-8 Relative abundance of fungal genera identified within the blood. Data are the relative abundance of the major bacterial taxa, characterized as having a mean abundance of >1% of the total fungal content in any one experimental group, identified in the plasma of rheumatoid arthritis (RA plasma, n = 1), ankylosing spondylitis serum (AS serum, n = 3), psoriatic arthritis plasma (PA plasma, n =2), PA serum (PA serum, n = 3), control serum (Control serum, n = 3), and control plasma (Control plasma, n = 3), samples as determined using amplification and sequencing of the ITS2. Data are mean abundance expressed as a percentage of the total bacterial sequence count, which were created by QIIME pipeline. (A) Taxa data grouped by the condition of fungal in blood, and (B) Taxa individual sample data of fungal in blood.

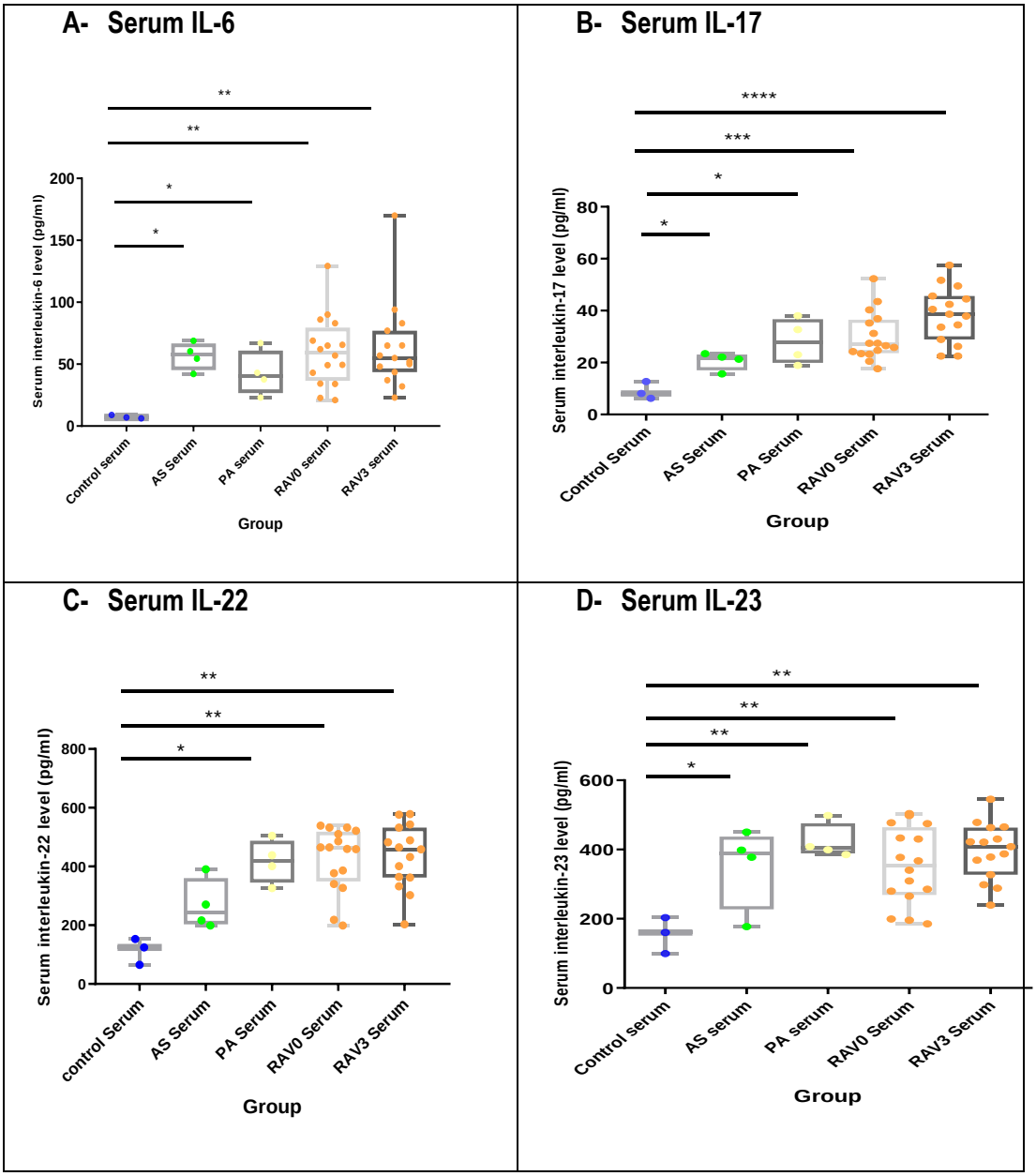
3.3.9 **Estimation of inflammatory cytokines in blood**

Serum and plasma levels of interleukin 6 (IL-6), 17A (IL-17A), 22 (IL-22), and 23 (IL-23) were measured using the Luminex system as described in the **Methods section, 2.4.1**. Levels of IL-6, IL-17, IL-22, and IL-23 were significantly elevated in serum and plasma of all patient groups and variables between disease conditions. We identified that serum and plasma levels of IL-6, IL-17, IL-22, and IL-23 were higher in RA patients relative to healthy controls. Moreover, IL-17, IL-6, and IL-23 were elevated in the serum of AS, serum, and plasma of PA patients compared to healthy subjects. Further, the level of IL-22 was significantly increased in serum and plasma PA patients relative to healthy controls **(Figure 3-9).**

Next, we investigated whether the plasma of RA patients have a different inflammatory status when compared to those with the RA serum V0 and V3, which may explain why the plasma RA did not cluster on bacterial microbiome analysis as expected in RA serum V0 and V3.

The Kruskal-Wallis test, with correction for multiple testing via the original FDR method of Benjamini and Hochberg method, revealed that the concentration of IL-6 was identified to be significantly decreased in the RA plasma patients **(RA plasma median (SD) = 30.9 (7.7) pg/ml)** in comparison to the RA serum V0 and V3 **(RA serum V0 and V3 median (SD) = 59.6 (28) pg/ml, 55 (35) pg/ml respectively)**. However, all other levels of inflammatory markers were unchanged between the RA plasma and RA serum V0 and V3 **(Figure 3-10).**

These results suggested that RA plasma were less inflamed when compared to RA serum V0, and V3 may clarify why the plasma RA did not cluster on bacterial microbiome analysis as expected in RA serum V0 and V3.



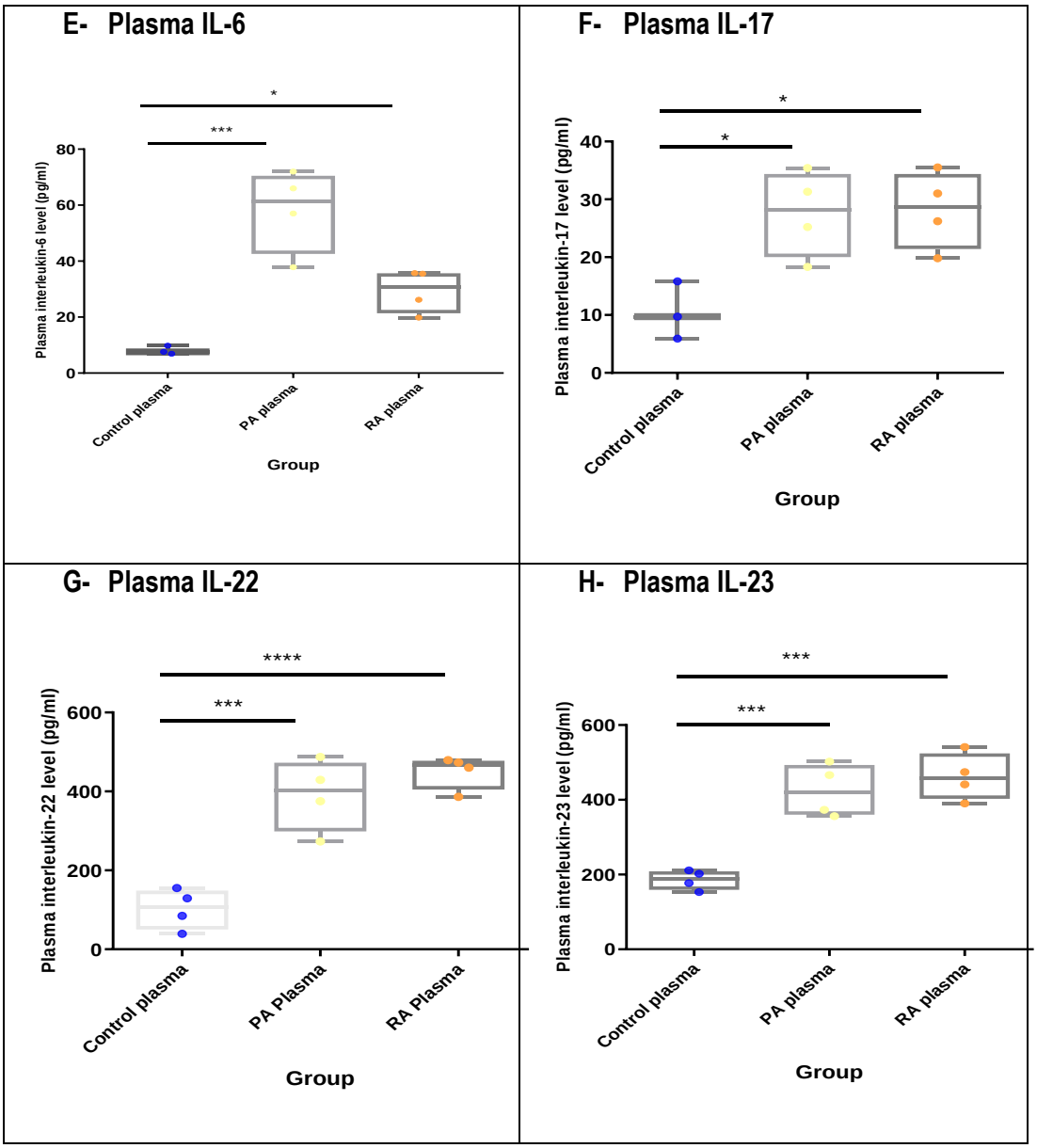


Figure 3-9 levels of IL-6, IL-17, IL-22, and IL-23 detected in the serum of ankylosing spondylitis serum and plasma of psoriatic arthritis, RA serum (RAV0 and RAV3) and plasma, and the serum and plasma healthy control subjects. Human Magnetic Luminex Screening Assay determines data. The box and whiskers show the distribution of data. From **A-D,** IL-6, IL-17, IL-22, and IL-23 levels were markedly increased in serum of rheumatoid arthritis (RAV0 and RAV3) patients compare to healthy controls serum. Further, IL-6, IL-17, and IL-23 levels were elevated in the serum of AS patients in comparison to controls. In addition, IL-6, IL-17, IL-22, and IL-23 were elevated in the serum of PA patients compared to healthy subjects. **From E-H,** IL-6, IL-17, IL-22, and IL-23 levels were significantly higher in both psoriatic arthritis and rheumatoid arthritis patients than healthy control. Data are a median of cytokine levels (pg/ml). The statistical significance between groups was determined by the Kruskal-Wallis test, with correction for multiple testing via the original FDR method of Benjamini and Hochberg method. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

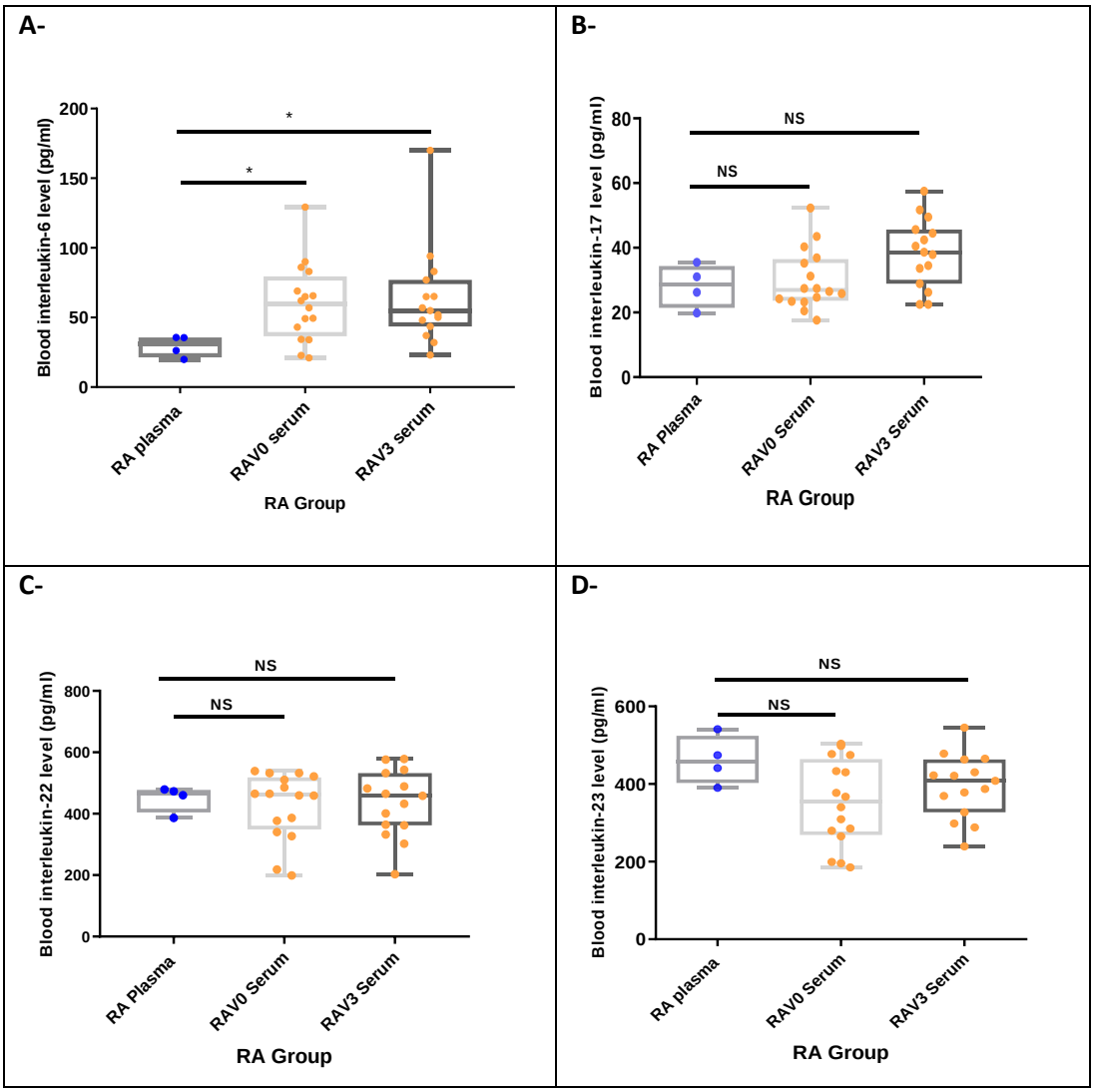


Figure 3-10 concentrations of IL-6, IL-17, IL-22, and IL-23 were identified in RA serum (RAV0 and RAV3) and plasma. Human Magnetic Luminex Screening Assay determines data. From **A**, the concentration of IL-6 was notably decreased in the plasma RA compared to the RA serum V0 and V3**. B-D,** There was no statistical difference in IL-17, IL-22, and IL-23 concentrations between the RA plasma and RA serum V0 and V3. Data are a median of cytokine levels (pg/ml). The statistical significance between groups was determined by the Kruskal-Wallis test, with correction for multiple testing via the original FDR method of Benjamini and Hochberg method. *P < 0.05.

3.3.10 **Changed cytokine responses in RA patients with alterations in taxonomic compositions of the serum microbiome**

We investigated whether genera significantly shifted via RA condition are correlated with increased specific inflammatory cytokine responses (IL-6, IL-17, IL-22, and IL23) to determine if taxa signatures are related to the degree of inflammation. The taxa include Halomonas, Shewanella, Streptococcus, Achromobacter, Escherichia-Shigella, Corynebacterium 1, Serratia*, Granulicatella, Gemella, and Staphylococcus.*

Spearman's rank correlation revealed that no such association was found to exist among Halomonas, Shewanella, Streptococcus, Achromobacter, Escherichia-Shigella, Corynebacterium 1, Granulicatella, Gemella, and Staphylococcus* with elevated inflammatory cytokines in the serum of RA patients. However, there was a negative correlation between *Serratia** and the elevation of IL-22 concentration in the serum of patients with RA ($r = -0.4$, $P = 0.03$), **See Table 3-, and Figure 3-11**.

We next investigated if the patients whose microbiome shifted following treatment had statistically different cytokine profiles (IL-6, IL-17, IL-22, and IL-23) to those whose microbiome did not shift the following treatment. T-test analysis found that no overall differences between the RA patients whose microbiome shifted following treatment and those whose microbiome did not shift the following treatment **(Figure 3-12).**

Table 3-4: Correlation of serum genera significantly changed via RA status with inflammatory cytokines (IL-6, IL-17, IL-22, and IL-23) is determined by Spearman's rank correlation coefficient.

Bacterial taxa vs. cytokine	Spearman's rank correlation	P value
Halomonas vs. IL-6	0.05	ns
Halomonas vs. IL-17	0.07	ns
Halomonas vs. IL-22	0.15	ns
Halomonas vs. IL-23	0.08	ns
Shewanella vs. IL-6	0.04	ns
Shewanella vs. IL-17	-0.1	ns
Shewanella vs. IL-22	-0.04	ns
Shewanella vs. IL-23	-0.13	ns
Streptococcus vs. IL-6	0.2	ns
Streptococcus vs. IL-17	-0.13	ns
Streptococcus vs. IL-22	0.08	ns
Streptococcus vs. IL-23	-0.2	ns
Achromobacter vs. IL-6	-0.03	ns
Achromobacter vs. IL-17	-0.1	ns
Achromobacter vs. IL-22	-0.01	ns
Achromobacter vs. IL-23	-0.1	ns
Escherichia-Shigella vs. IL-6*	-0.1	ns
Escherichia-Shigella vs. IL-17*	-0.08	ns
Escherichia-Shigella vs. IL-22*	-0.1	ns
Escherichia-Shigella vs. IL-23*	-0.2	ns
Corynebacterium 1 vs. IL-6	-0.01	ns
Corynebacterium 1 vs. IL-17	-0.1	ns
Corynebacterium 1 vs. IL-22	-0.09	ns
Corynebacterium 1 vs. IL-23	-0.06	ns
Granulicatella vs. IL-6	0.12	ns
Granulicatella vs. IL-17	-0.2	ns
Granulicatella vs. IL-22	-0.2	ns
Granulicatella vs. IL-23	0.06	ns
*Serratia** vs. IL-6	-0.1	ns
*Serratia** vs. IL-17	0.3	ns
*Serratia** vs. IL-22	**-0.4**	**0.03***
*Serratia** vs. IL-23	0.2	ns
Gemmella vs. IL-6	0.09	ns
Gemmella vs. IL-17	-0.05	ns
Gemmella vs. IL-22	-0.03	ns
Gemmella vs. IL-23	-0.05	ns
Staphylococcus vs. IL-6	0.3	ns
Staphylococcus vs. IL-17	-0.3	ns
Staphylococcus vs. IL-22	0.2	ns
Staphylococcus vs. IL-23	-0.2	ns

ns= not statistically significant, *P < 0.05

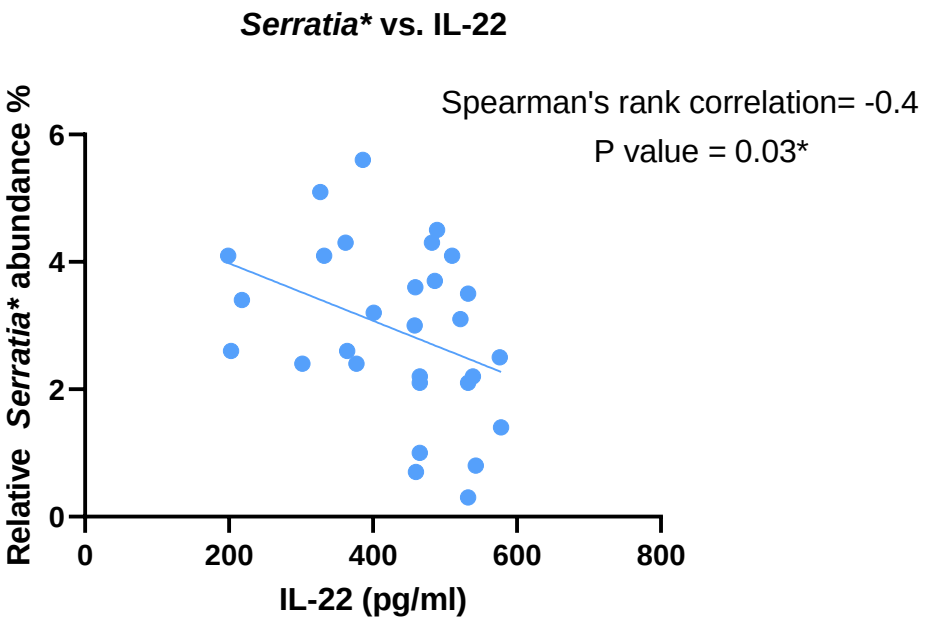
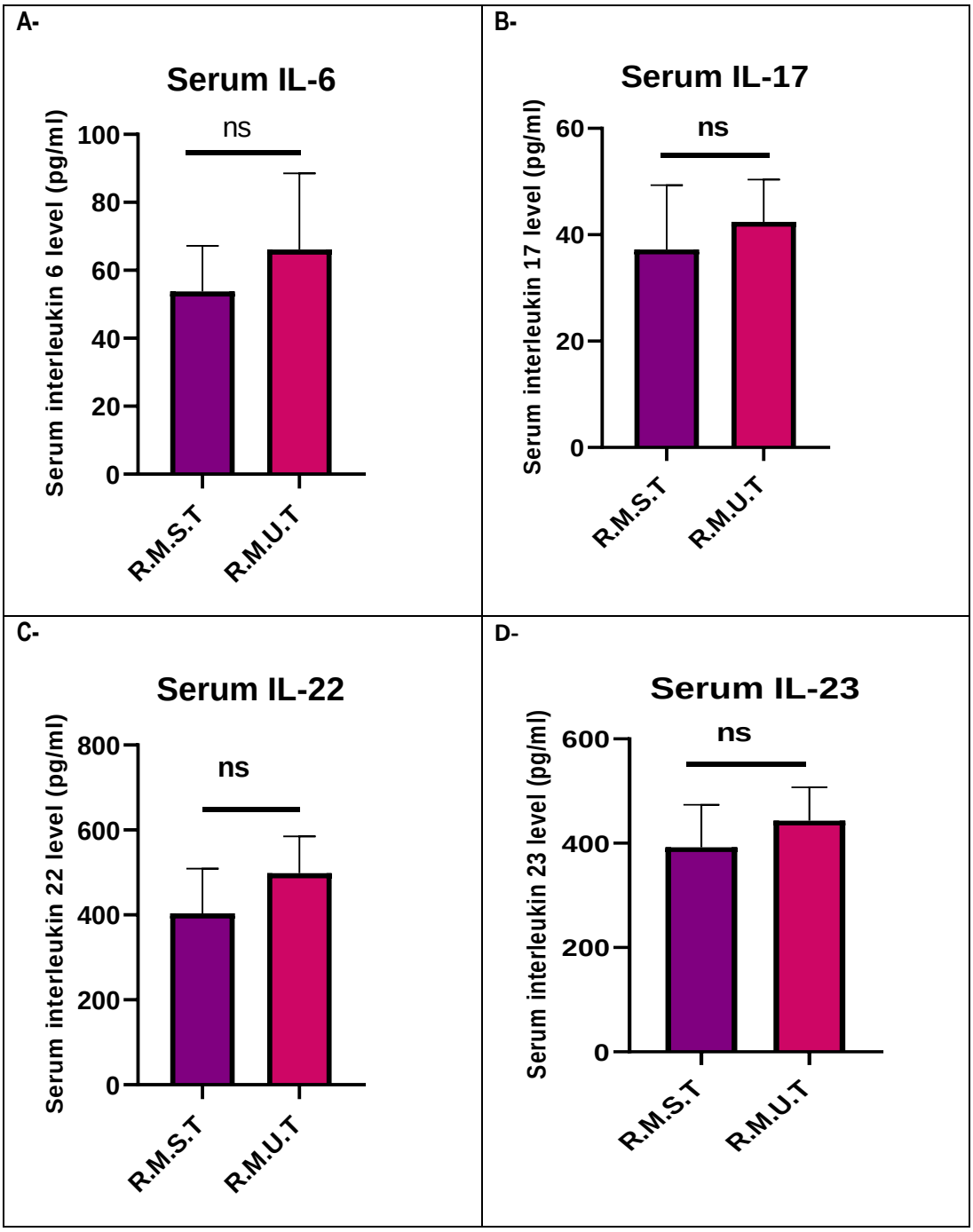


Figure 3-11 Correlation between relative abundances of *Serratia and the elevation of IL-22 concentrations detected in the sera of patients with RA**. Relative abundances of *Serratia** negatively correlates with the elevation of IL-22 concentrations (r=-0.4, P=0.03) in the serum of patients with RA. Data were statistically analysed using the Spearman correlation test. The level of statistical significance was set at a 95% confidence interval ($p < 0.05$), and the statistical analysis was determined using Prism 8.0 software. *P < 0.05.



R.M.S.T RA patients whose microbiome shifted following treatment
R.M.U.T RA patients whose microbiome unshifted following treatment

Figure 3-12 Human Magnetic Luminex Screening Assay measured IL-6, IL-17, IL-22, and IL-23 levels in serum of the RA patients whose microbiome shifted after treatment, and those whose microbiome did not shift the following treatment. A-D, IL-6, IL-17, IL-22, and IL-23 levels were not significantly changed between the RA patients whose microbiome shifted following treatment and those whose microbiome did not shift the following treatment. Values represent the mean (SD) of cytokine levels (pg/ml). The statistical significance between groups was determined by T-test, ns= not statistically significant.

3.4 Discussion

Emerging evidence suggests that microbiome dysbiosis in the gut, oral, lung, and urinary tract of RA patients may play a role in the initiation and development of RA illness. On the other hand, ankylosing spondylitis (AS) and psoriatic arthritis (PA) appear to have different pathogenesis as microbiome dysbiosis in the gut of patients with AS and PA was observed (Gilis *et al.*, 2018).

The existence of viable organisms in the blood of a human cohort is a continuingly controversial subject matter (Castillo *et al.*, 2019). However, several studies have presented evidence for the presence of entire microorganisms utilising a range of different techniques (Whittle *et al.*, 2019). Previous studies involving PCR-based methods have shown the existence of microbial DNA (Martinez-Martinez *et al.*, 2009), while the uncertainty remains about the origin of the microbiome DNA detected in these studies (Hornung *et al.*, 2019). It has been hypothesised that leaching of microbial DNA from classical microbial niches happen in both health and illness, and such alterations in the circulatory system mainly report upon the composition of classical niches (for instance, the gut and oral, See mechanisms in Chapter 1); this is known to be perturbed in illness (Loyola-Rodriguez *et al.*, 2010; Potgieter *et al.*, 2015). There is an alternative hypothesis that claims that the DNA found in the blood could originate either from a dormant or L form of bacteria (Kell and Pretorius, 2015; Markova, 2017).

No information is available about the association blood dysbiosis and RA, AS, and PA illnesses. More research effort is required to line out the type changes in the blood microbiome and investigate how it does contribute to the RA, AS, and PA pathogenesis. To this end, we characterised the blood microbiome of patients with rheumatoid arthritis (RA),

ankylosing spondylitis (AS), and psoriatic arthritis (PA), in comparison with healthy control subjects, and we try to identify specific microbiome signatures associated to RA, AS, and PA illnesses. This evidently will increase our understanding of pathogenesis and or reveal a pool of candidate biomarkers for further development. Furthermore, we evaluated the correlation of inflammatory cytokines (IL-17-A, IL-22, IL23, and IL-6) with these inflammatory conditions, as it has been proved that these cytokines levels may increase within arthritic conditions. An example includes the increase in IL-6 in the serum of patients with RA (Srirangan and E. H. Choy, 2010; Yoshida and Tanaka, 2014a; Narazaki, Tanaka, and Kishimoto, 2017a; Boyapati *et al.*, 2019). Furthermore, our findings also showed how the microbiome changes post-treatment of RA patients.

Principal coordinates analysis (PCoA) is ordination techniques, which is used to decrease the dimensionality of microbiome data sets so that a summary of the beta diversity relationships can be visualised in two- or three-dimensional scatterplots (Goodrich et al., 2014). The PCoA, each of which explains a certain fraction of the variability, seen in the data set, are plotted to create a visual representation of the microbial population compositional differences among samples (Goodrich *et al.*, 2014). Observations based on PCoA plots can be substantiated with statistical analyses that evaluate the clusters.

We used this technique to reduce the complex, multidimensional data for visualisation of patterns and to assess whether the blood microbial community could be differentiated between diseased disorders and healthy controls.

Our findings of the PCoA plots identify that the types of microbiome present in the Sera Laboratories and Haywood hospital samples are very different. There is also a wide variation across the RA serum samples.

There are a number of reasons that could explain the differences in our sample sets, such as age, dietary patterns, and gender.

Population-based studies have identified multiple factors that associate with the showed variation in the human microbiome composition. They include age, dietary patterns, gender, body mass index, disease state, and disease severity (Yatsunenko *et al.*, 2012; Huang and Boushey, 2015; Mar *et al.*, 2016; Nibali and Henderson, 2016; Kho and Lal, 2018; Hasan and Yang, 2019).

The age strongly affects human health, partly by modulating intestinal microbiome composition (Nagpal, Mainali, *et al.*, 2018). Intestinal microbiota do not age per se, but the incidences of comorbidities associated with intestinal microbes tend to rise as the host grows older (Nagpal, Mainali, *et al.*, 2018); even though it remains unclear whether microbiota modifications are cause or consequence of host ageing (Nagpal, Mainali, *et al.*, 2018). It is found that older people have a different intestinal microbiome profile in comparison to healthy people (Nagpal, Mainali, *et al.*, 2018). Generally, the diversity of the gut microbiome and the carriage of commensals such as *Bifidobacteria, Bacteroides,* and *lactobacilli* are identified to be decreased, while the levels of opportunists such as *C. perfringens, enterobacteria, and C. difficile* are raised in the elderly (Nagpal, Mainali, *et al.*, 2018). This variation could be attributed to several reasons associated with senescence, such as changed lifestyle, lesser mobility, dietary schedule, weakened immune strength, reduced intestinal and overall functionality, recurrent infections, changed gut morphology, and use of medications (Nagpal, Mainali, *et al.*, 2018).

It is well established that the diet can affect the human microbiome (Singh *et al.*, 2017). Research on mice has revealed that shifting from a fiber-and antioxidant-rich Mediterranean nutrition to a Western diet heavy in fat and protein can change the

microbiome's community within a day (Singh *et al.*, 2017). Furthermore, diets high in sugar can reduce microbiome diversity within seven days—a change that has been associated with some inflammatory conditions such as diabetes (Singh *et al.*, 2017). Researchers have also found that antibiotics or antibacterials can knock down or disrupt the human body's microbiome in ways that could encourage disease (Langdon, Crook, and Dantas, 2016).

Gender is one of the significant variables influencing the microbiome, but the association has not yet been adequately examined. Although the results are inconsistent, a number of investigations have shown gender differences in the gut microbiome (Kim *et al.*, 2020).

In a USA research conducted on a mainly Caucasian people, the microbiome of the women was characterised via a lower abundance of phylum Bacteroidetes (Dominianni *et al.*, 2015). Research in Italy revealed that the mucosa-associated microbiome was varied between men and women (Borgo *et al.*, 2018). The mucosa-associated microbiome in women revealed a higher abundance of *Streptococcaceae, Lactobacillales, Actinobacteria, Bifidobacterium, and unclassified Clostridia*. At the species level, *Bifidobacterium adolescentis* was associated with the women and *Gemmiger formicilis* with the men (Borgo *et al.*, 2018).

Research of Chinese family members, which was conducted utilising group-specific DGGE profiling of *Bacteroides* taxa, a higher abundance of *Bacteroides thetaiotaomicron* was found in the men (Li *et al.*, 2008).

In a study conducted via four centres in Germany, France, Italy, and Sweden, a higher abundance of the *Bacteroides-Prevotella taxa* was identified in the men (Mueller *et al.*, 2006)

In subgroup analysis, this gender difference in the gut microbiome was not found in healthy people, whereas it was evident in infected individuals with ten times more different microbial characteristics than that in healthy people (Singh and Manning, 2016). Besides the microbial structure and diversity, a recent study revealed that sex was associated with the functional gene richness of the colon (Zhernakova *et al.*, 2016).

Gut microbiota may differ between males and females, and that the grade of obesity may influence these differences. The divergence in intestinal microbiota observed between males and females might have a role in the definition of gender differences in the prevalence of metabolic and intestinal inflammatory conditions (Haro *et al.*, 2016). The study by Haro et al. (Haro *et al.*, 2016) proposes that the microbiome composition may differ between males and females and that the grade of obesity may influence these differences. The alteration in microbial community observed between men and women might have an influential role in the definition of gender differences in the prevalence of intestinal inflammatory and metabolic diseases (Haro *et al.*, 2016).

Gender differences in the evolution and presentation of different diseases have been known, but the associated mechanism is unclear (Danska, 2014a). Gender differences in the intestinal microbiome may play a role in the gender differences in conditions (Danska, 2014a).

The gender differences in the innate and adaptive immune systems are well understood (Klein and Flanagan, 2016). Receptors for sex hormones are expressed on most immune cells, and thus sex hormones may play a role in establishing the gender difference in the immune response (Elderman, de Vos and Faas, 2018). Because the gut microbiome interacts with the host immune system, it can be expected that the gender differences in the intestinal microbiome have some role in the gender differences in immunity (Fransen *et*

al., 2017). NOD mice exhibit spontaneous, immune-mediated loss of their pancreatic beta cells, producing in type 1 diabetes mellitus. A higher incidence of diabetes mellitus has been found in female SPF NOD mice than that of males (Yurkovetskiy *et al.*, 2013).

Interestingly, this sex difference was not shown in GF NOD mice. In contrast, it appeared again after the colonisation of gut microbiotas that are identified to be associated with the sex difference in SPF NOD mice (Yurkovetskiy *et al.*, 2013). In research of patients with encephalomyelitis/chronic fatigue syndrome, another instance of an immune-related disease, there was no difference in the overall microbiome composition between the genders (Yurkovetskiy *et al.*, 2013).

Nevertheless, the abundance of *Clostridium, Streptococcus, Lactobacillus, and Bifidobacterium,* according to a specific symptom, are different between sexes (Wallis *et al.*, 2016).

Intestinal inflammation might also have gender differences about the gut microbiome. In a mouse model of colitis induced with 2, 4, 6-trinitrobenzene sulfonic acid, the males showed more severe colonic inflammation (Kozik *et al.*, 2017). The FMT animal model of another study showed that female recipients lost significantly more weight after taking the male microbiome when compared to those with the weight after taking the female microbiota, proposing that the male microbiome caused more gut inflammation (Fransen *et al.*, 2017).

Probiotics also caused different inflammatory responses from female and male mice (Lee *et al.*, 2017). In female Wistar mice presented to water avoidance stress, the administration of Lactobacillus farciminis significantly lowered the colonic mucosal mast cell count and decreased the levels of inflammatory cytokines only in the female mice (Lee *et al.*, 2017). Besides, sex differences in response to probiotic Lactobacillus animalis NP-51

administration were recorded for cytokine responses, intestinal metabolic profiles, and intestinal microbiome in Mycobacterium-treated mice (Karunasena *et al.*, 2014)

It has been reported that the change of microbiome compositions are associated with the development of several human diseases such as RA, ankylosing spondylitis, psoriatic arthritis, and obesity (Ding *et al.*, 2019). Further, the severity of some diseases such as RA, asthma, and obesity, contribute to exaggerated inflammatory and microbiome changes (Horta-Baas *et al.*, 2017; Michalovich *et al.*, 2019).

Blood samples from 44 subjects were analysed in this study, and our analyses revealed the presence of a complex blood bacterial and fungal community in health and disease. At the phylum level, four key phyla dominated our blood samples; *Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes.*

These results mirror previous studies (Amar *et al.*, 2013; Païssé *et al.*, 2016; Olde Loohuis *et al.*, 2018; Whittle *et al.*, 2019) and further support the notion of a core blood microbiome predominated by four key phyla. Proportions of several genera found in this study have previously been found in the blood of healthy human subjects as part of a separate investigation (Whittle *et al.*, 2019), although in differing proportions. Moreover, other investigations have also shown these genera in different proportions among both illnesses and healthy subjects.

The *genera Halomonas and Shewanella* were selectively abundant in the serum of RA patients (RA V0 and RA V3) compared to control subjects. It has been seen that the ability of *Halomonas species* to grow at high salt concentrations. Recently, *Halomonas* has a role in a limited number of human infections, including dialysis-related (Kim, Lee, and Stevens, 2013). *Halomonas* has been identified in a range variety of human samples including, the gut (Seck *et al.*, 2016), bronchoalveolar lavage fluid (Sverrild *et al.*, 2017), and skin of

healthy human (E. A. Grice *et al.*, 2008), and also *Halomonas* found in the blood of vertebrates, including rodents (Cohen *et al.*, 2015). It has also been detected in the synovial fluid of Tunisian patients with reactive and undifferentiated arthritis (Siala *et al.*, 2008).

Further, alterations in the abundance of genus *Halomonas* has been shown in bronchoalveolar lavage fluid of asthmatic patients with lower eosinophilic counts (Sverrild *et al.*, 2017). Another study it has been observed that *Halomonas* was positively correlated with obesity in the gut microbiome of rodents (Waldram *et al.*, 2009). Furthermore, identification of *Halomonas* in the salivary microbiome has been linked with inflammatory markers such as IL-1β (Acharya *et al.*, 2017a), which is a critical inflammatory cytokine also related to RA, supports the notion that *Halomonas* and inflammation are linked.

However, *Halomonas* has been found as a potential contaminant due to its existence in negative control samples, which subject to sequencing (Santiago *et al.*, 2016; Whittle *et al.*, 2019). It should be noted that only six reads mapping to the genus *Halomonas* were identified in the single negative control, which returned to sequence data, in comparison to a mean of 3500 reads found in our experimental samples; therefore, contamination with this taxon in our samples was not expected.

Shewanella species are found in seawater, and are increasingly linked with human infections, and regarded as a reservoir for antimicrobial resistance. Many people infected with these microorganisms report prior exposure to seawater (Yousfi *et al.*, 2017). *Shewanella species* colonise and survive in the *human gut*. These species are perturbed in the human gut microbiome in different physiological and illness conditions (Rojo *et al.*, 2017; Smid *et al.*, 2018). *Shewanella* has been found in the blood microbiome of healthy people subjects too, in particular, associated with RBCs (Païssé *et al.*, 2016).

Moreover, it has been reported that *Shewanella* causes soft tissue infection in patients with RA and diabetes (Tsai *et al.*, 2008). Regardless of other studies finding the *Shewanella* genus as a potential contaminant, we found no evidence of such in our negative control reactions.

Interestingly, both genera *Halomonas* and *Shewanella* were identified to be elevated in RA patients and are salt tolerant.

Furthermore, the abundances genera *Halomonas* with *Shewanella* microbiome were significantly higher in the gut of patients with inflammatory conditions, for example, Idiopathic pulmonary fibrosis (D'Alessandro-Gabazza *et al.*, 2018), asthma (Sverrild *et al.*, 2017), Keratitis (Jayasudha *et al.*, 2018), and Uveitis (Jayasudha *et al.*, 2018).

Depended on the sodium permeation-changing ability of *Shewanella* (Wang *et al.*, 2008), and the halophilic and pro-apoptotic properties of *Halomonas* (Martínez-Cánovas *et al.*, 2004; Ruiz-Ruiz *et al.*, 2011; Sagar *et al.*, 2013; D'Alessandro-Gabazza *et al.*, 2018), it has hypothesised that the existence of *Shewanella* increases extracellular levels of salt by preventing intracellular passage of sodium and thereby generating a favourable microenvironment for growing *Halomonas* (D'Alessandro-Gabazza *et al.*, 2018).

These halophilic microorganisms excrete potent pro-apoptotic factors that may stimulate and consequently encourage increased apoptosis of alveolar epithelial cells (Ruiz-Ruiz *et al.*, 2011; Sagar *et al.*, 2013; D'Alessandro-Gabazza *et al.*, 2018). Activated epithelial cells express several transforming growth factors, including TGF-β1, which may further activate the growth of *Halomonas* through increasing extracellular salt production through blocking of cell membrane expression of Na and Cl channels (Frank *et al.*, 2003; Peters *et al.*, 2014; Kabir *et al.*, 2018).

Many researchers reported high expression of TGF-β1 and its inflammatory role in patients with asthma (Tirado-Rodriguez *et al.*, 2014), Keratitis (Roux *et al.*, 2016), Idiopathic pulmonary fibrosis (D'Alessandro-Gabazza *et al.*, 2018), and Uveitis (Ooi *et al.*, 2006).

The following considerations support the eventual harmful role of a salty microenvironment in the pathophysiological situation of several inflammatory diseases such as Idiopathic pulmonary fibrosis:

(1) Increased circulating sodium chloride enhances TGF-β1 expression (Hovater and Sanders, 2012).

(2) Salt reduces the protecting activity of mucin (Travis *et al.*, 1999). The point that mucin, in turn, can prevent the adverse effects of salt (Travis *et al.*, 1999) may clarify why Idiopathic pulmonary fibrosis patients with a common risk polymorphism in Mucin 5B (major gel-forming mucin in the lung that plays a fundamental role in mucociliary clearance and host defence), associated with increased mucin production, have significantly enhanced survival (Peljto *et al.*, 2013).

(3) Acute exacerbation of the condition in Idiopathic pulmonary fibrosis patients is apparent following diagnostic bronchoalveolar lavage methods in which a high volume of saline is utilised ((Sakamoto *et al.*, 2012).

Recently, Sodium Chloride microenvironment has been proposed as aggravates RA by Th17 Polarization present in the spleen of the CIA mouse through the induction of pathogenic CD4+ T helper cells that generate interleukin-17 (Th17 cells) (Jung *et al.*, 2019).

Further, Liao et al. have found both *Shewanella* and *Halomonas* to be elevated in the gut microbiome of mice nourished with chondroitin sulphate (Liao *et al.*, 2017). This has been

associated with gut inflammation and the inflammation of the joint, leading the authors to the assumption that the change in the intestinal microbiome participates in the pro-inflammatory condition (Liao *et al.*, 2017).

Moreover, D'Alessandro-Gabazza and his workers hypothesised that changes in sodium permeation due to the existence of *Shewanella species* stimulate the growth of *Halomonas* species (D'Alessandro-Gabazza *et al.*, 2018). As a number of studies have identified these two genera to live together, our results cannot be disregarded as incidental or contaminants.

Achromobacter, Escherichia/Shigella, Serratia*, Corynebacterium-1, Streptococcus, Granulicatella, Staphylococcus*, and *Gemella* were the genera significantly decreased in abundance in the serum of RA patients relative to control subjects. *Achromobacter* has been recently identified in the blood of asthmatic patients and healthy controls (Whittle et al., 2019). Recent studies have reported that *Achromobacter* and to a lesser extent *Serratia** are considered as lymphoid tissue-resident commensal (LRC) bacteria in humans and animals (Tatro et al., 2014; Fung et al., 2016a). The LRC bacteria can control their growth out of IL-22 to regulate systemic inflammation (Fung et al., 2016b). Therefore, a decrease in these bacterial DNA in the serum of RA patients could be indicating a pro-inflammatory condition. The genera *Gemella, Granulicatella*, and *Streptococcus* were found in the saliva of the healthy people subjects (Acharya et al., 2017b). It has been identified that the taxa *Gemella, Granulicatella,* and *Streptococcus* were significantly less abundant in the subgingival microbiome of RA patients (Lopez-Oliva *et al.*, 2018), supported their presence in classical microbiome niches. Members of the genera *Staphylococci* and *Corynebacterium* are accepted as members of the normal human microbiome of the skin, oral cavity, and the gut. Recently, these genera have been identified in the blood of healthy people (Païssé et al., 2016). Therefore, their decrease, as

we have observed in the blood microbiome in serum RA patients, along with the other alterations mentioned above, indicates the occurrence of dysbiosis in these distant locations, reflected in the abundance of DNA that reaches the blood, and support notion blood microbiome may play a critical role in RA disease.

One of the main findings of our study was that the serum microbiome of 13/17 patients with RA progressed towards the "healthy microbiome" after induction of treatment. The shift was not influenced by treatment modality.

There provides evidence that the microbiome of various locations, such as the gut and oral does partly normalise with treatment in RA patients (Zhang *et al.*, 2015; Picchianti-Diamanti *et al.*, 2018). However, another study by Beyer K. et al. (Beyer *et al.*, 2018) found that there was no relationship between the microbiome diversity in the oral of RA patients and the use of DMARDs. Several factors might be associated with this. It might have been due to different study designs, sampling locations, or techniques applied. While this effect was not linked with any one specific treatment modality, 3/4 of the patients with a 'non-responsive serum microbiome' had seropositive RA, at the same time as the majority (77%) of those who responded were seronegative. Older studies have shown the alteration in oral and intestinal microbiome in RA patients when treated with MTX and Etanercept (Zhang *et al.*, 2015; Picchianti-Diamanti *et al.*, 2018). While several studies do not differentiate between treatment response and seropositivity, a recent study showed that seronegative RA might show a better response to treatment, supporting our results (Choi and Lee, 2018). Our results have shown that there was a better normalisation of the serum microbiome among the seronegative RA cohort. A recent study has shown the significance of the intestinal microbiome in drug metabolism. Increasing evidence proposes that

differences in the intestinal microbiome population may demonstrate individual drug responses (Zimmermann *et al.*, 2019).

These results have suggested that the microbiome of RA patients appeared to change following treatment. The shift was not influenced by treatment modality and, therefore, would seem more likely that the microbiome is reflective inflammatory status rather than microbiome being causative. Furthermore, the modulating the microbiome response following the commencement of treatment detected with those patients with RF/CCP negative RA more likely to progress toward a control microbiome community than those who are RF/CCP positive.

In the main, the taxa found as disease or treatment responsive have been previously described as common inhabitants of the human microbiome, more commonly identified in the oral cavity and gut. The abundance of the genera *Haemophilus, Alloprevotella, Eremococcus*, and *Lachnospiraceae_UGC-001* increased significantly with treatment and contributed to the normalisation of the microbiome. *Haemophilus* species are microorganisms that colonise in the human mucous membranes of the gut, mouth, upper respiratory tract, and vagina (Tortora, Case, and Funke, 2016). *Haemophilus species* have been indicated to be less abundant in the gut and oral microbiomes of RA patients relative to unrelated healthy control, and their abundance is partly normalised after treatment (Zhang *et al.*, 2015; X. Wu *et al.*, 2016). *Lachnospiraceae UCG-001* belonging to the *Lachnospiraceae* family was found these taxa in previous human and animal investigations to be elevated in RA individuals (Liu *et al.*, 2016; X. Wu *et al.*, 2016). A significant Higher in genus *Lachnospiraceae UCG-001* was indicated in the colon of Sirt3 knockout mice with a range broad antibiotic cocktail (ampicillin, vancomycin, metronidazole, and neomycin) compared to Sirt3 knockout mice without treatments (Zhang *et al.*, 2018). *Alloprevotella* species were correlated with early RA independent of the severity of periodontitis (Wolff *et*

al., 2017), whereas we identified this taxon to be reduced in RA in comparison to the healthy condition. Genus *Eremococcus* has been previously identified in the genital tract of horses (Collins *et al.*, 1999; Wittenbrink, 2012).

Furthermore, *Eremococcus* has been seen in human blood (Lorenz, Mühl and Disqué, 2015), while there is no link identified between *Eremococcus* and RA illness to date. However, our results were not in full agreement with enriched/depleted taxon indicated at different body locations in RA in previous studies; one must mention that our analyses are conducted in the blood, which possibly harbours microbial DNA from a range of niches. On this basis, these findings certainly warrant further studies.

In considering the fungal microbiome, fungal ITS2 gene was not identified in the serum of RA patients (RA V0 and RA V3). However, the fungal ITS2 was detected in the serum of 3 of out 4 (75%) patients with AS, 1 of 4 (25%) plasma with RA, 2 out of 4 (50%) PA plasma, 3 out of 4 (75%) PA serum, 3 out of 4 (75%) healthy control subjects' serum, and 3 out of 4 (75%) in the plasma of healthy control subjects. The dominant fungal phyla in the blood of ITS2 were *Basidiomycota* and *Ascomycota*. These results are in agreement with another study which has found the *Basidiomycota* and *Ascomycota* phyla are predominant in the blood of healthy human subjects (Panaiotov, Filevski, Equestre, Nikolova, Kalfin and Panaiotov, 2018), and further support the notion of a core blood fungal microbiome predominated by two phyla.

At the genus level, our blood samples were dominated by the genus *Malassezia. Malassezia* was abundant in the blood of diseased and healthy control volunteers. Malassezia is ubiquitously existent on human skin (Limon, Skalski and Underhill, 2017), gastrointestinal (Hallen-Adams and Suhr, 2017), and oral (Dupuy *et al.*, 2014), and acts as

an opportunistic pathogen. We identified no statistically significant differences in the blood fungal population between diseased groups and healthy control subjects.

Levels of IL-6, IL-17, IL-22, and IL-23 were abundantly present in serum and plasma of all patient groups and variable between illness conditions. We identified that serum and plasma levels of IL-6, IL-17, IL-22, and IL-23 are higher in RA patients relative to healthy controls. Interleukin 6 (IL-6) has a fundamental role in the pathophysiology of rheumatoid arthritis (RA). It is indicated in abundance in the serum and synovial fluid of RA patients, and the level associated with the illness activity and joint destruction (Srirangan and E. H. Choy, 2010; Yoshida and Tanaka, 2014a; Narazaki, Tanaka and Kishimoto, 2017a; Boyapati *et al.*, 2019). IL-6 can stimulate synovitis and joint destruction via activation of osteoclast maturation, neutrophil migration, and vascular endothelial growth factor (VEGF)-stimulated pannus proliferation (Srirangan and E. H. Choy, 2010). A transient synthesis of IL-6 has a role in host defence against infectious illnesses and tissue injuries via activation acute phase reactions and immunological and hematopoietic responses (Yoshida and Tanaka, 2014a). However, dysregulated persistent production of IL-6 could lead to the evolution of several immune-mediated illnesses. IL-6 may also be mediating many of the systematic manifestations of RA involving anaemia through hecipidin production, activation of the acute-phase reaction [including C-reactive protein (CRP)], and osteoporosis from its effect on osteoclasts (Srirangan and E. H. Choy, 2010). IL-6 may participate in the stimulation and maintenance of the autoimmune process via TH-17 differentiation and B-cell maturation (Eto *et al.*, 2011).

IL-17 is the signature cytokine of the newly-defined “Th17” T helper cell population and has been associated with the pathogenesis of various inflammatory illnesses such as in RA (Gaffen, 2009a). The present study revealed a significantly higher level of serum and plasma IL-17A levels in RA patients than in healthy control subjects. The data revealed by

several authors in the literature agreed with our findings. Kohno et al. (Kohno *et al.*, 2008) reported that the level of IL-17 gene expression in peripheral blood mononuclear cells from RA patients was considerably higher than healthy control subjects. Melis et al. (Melis *et al.*, 2010) found a high serum IL-17 level in RA patients. Moreover, serum IL-17 level was increased in RA patients relative to healthy controls (Metawi *et al.*, 2011). Furthermore, studies in RA animal models, mammalian cell culture systems support a role for IL-17 in stimulating RA (Gaffen, 2009a).

It has been indicated that IL-22 is elevated in the serum of RA patients and related to erosive illness and might serve as a marker for joint destruction in the RA cohort (da Rocha *et al.*, 2012a; Jan Leipe, 2016). Further, elevated serum IL-22 allows discrimination between patients with different radiographic progression and also indicates a potential involvement of IL-22 in the pathophysiology of RA, mainly in patients with RF antibodies and long term illness (Leipe *et al.*, 2011; da Rocha *et al.*, 2012a; Jan Leipe, 2016).

Several studies support the role of IL-23 in RA patients. For instance, Kim et al. identified that IL-23 levels in serum and synovial fluid are higher in RA patients than in OA patients or healthy controls, and IL-23 may be a valuable biomarker for the diagnosis of RA (H.-R. Kim *et al.*, 2007). IL-23 is a proinflammatory cytokine that participates in the development and maintenance of Th17 cells in inflammatory autoimmune illnesses. Furthermore, IL-23 affects the pathogenesis of inflammation and joint destruction by interaction with other cytokines; for instance, IL-17 and TNF-α (Yago *et al.*, 2017).

In our study, we have found that the levels of IL-17, IL-6, and IL-23 were elevated in the serum of AS, serum, and plasma of PA patients compared to healthy subjects. Further, the level of IL-22 was significantly increased in serum and plasma PA patients relative to healthy controls. This is in agreement with other studies (Yasumoto, Imayama and Hori,

1995; Elkayam *et al.*, 2000; Mei *et al.*, 2011; Chen *et al.*, 2012; Ogata, Kumanogoh and Tanaka, 2012; Przepiera-Będzak, Fischer and Brzosko, 2015; W. Liu *et al.*, 2015; Wang *et al.*, 2017; Blauvelt and Chiricozzi, 2018). In our opinion, this confirms the previously proposed role of IL-6, IL-17, IL-6, and IL-23 in AS and PA. It will be essential to conduct further investigations on the possibility of utilising antibodies against these cytokines in selected AS and PA patients that may help to manage these illnesses.

Relative abundances of Serratia* negatively correlates with the elevation of IL-22 concentrations in the serum of patients with RA.

As mentioned above, *Serratia** has been found as lymphoid tissue-resident commensal bacteria in humans and animals (Fung, Artis, and Sonnenberg, 2014; Fung *et al.*, 2016a). There was an association between lymphoid tissue-resident commensal bacteria and IL-22 in regulating inflammatory response, whereas they control their growth through IL-22 (Fung, Artis, and Sonnenberg, 2014; Fung *et al.*, 2016a). Thus, a lower percentage of *Serratia** may induce a high production of IL-22 in the serum of RA patients.

There are many significant limitations in this study that could be addressed in future research. Firstly, the study used a small number of healthy control samples because of the small number of cohorts size were provided from our sources. A small control sample size may not provide sufficient statistical power to identify a difference between diseased and control groups (i.e., low power) (Hutchins *et al.*, 2015).

Secondly, the control group subjects were obtained from a different lab to the V0/V3 data. The different locations of donors might lead to the different microbiome patterns seen (Shin *et al.*, 2016). It is possible, therefore, that some differences in their microbiome composition could be explained by variables for which data were not available for our

procured material, differences in collection technique, or even the immediate environment at the time of collection.

However, we strictly controlled all analytical parameters from the moment of sample acquisition; our sample cohorts also came as blood product samples in sterile tubes. Further, all samples were processed in parallel and in a randomised order to avoid the introduction of batch effects.

Additionally, factors that could affect the human microbiome, such as gender, age, and BMI, were not investigated, which might explain microbiome variation between the RA patient populations in PCoA. However, we could not be assessed entirely effect these factors on our patient's samples due to the lack of patient information from our source.

We are aware that a range of factors changes the microbiome, and such analyses are highly sensitive to pre-analytical conditions. While we were unable to practically address these limitations herein given the retrospective nature of our study design, we support strongly for further prospective studies in this area, which control pre-analytical conditions inclusive of the sample collection phase.

Through amplification and sequencing of the bacterial 16S rRNA and ITS2 genes, we describe the presence of a blood bacterial and fungal microbiome in patients with RA, AS, PA, and healthy control subjects. RA disease state is associated with bacterial blood dysbiosis, and a further, the bacterial microbiome of RA patients appeared to change following treatment. Moreover, the modulating the microbiome response following the commencement of treatment detected within those RA patients with RF/CCP negative rather than RF/CCP positive.

In the main, the taxa identified as disease or treatment responsive have been previously described as common inhabitants of the human microbiome, more commonly observed in

the gut and oral cavity. These findings support our developing hypothesis that microbial DNA found within the blood translocates from more classical microbiome niches such as the gut, and oral those are undergoing disease association perturbation. These blood-derived signatures may have significant utility as the disease. Further studies are required to investigate these preliminary findings.

3.5 Conclusion

This study reports that the presence of a blood microbiome in illnesses and health, and determine specific bacterial taxa altered in RA disease and following treatment. It is likely the microbiome originates from one of the classical microbiome niches (the gut, mouth, urogenital tract, skin) and reaches the circulation. We also identify the microbiome of RA patients appeared to normalise following treatment partially. These findings may have an essential use as disease biomarkers and propose this area warrants further investigation.

Chapter 4

4 Characterisation of the synovial fluid microbiome of RA and healthy control subjects

4.1 Overview

The presence of microbiome DNA in places usually considered to be sterile of the human body, such as synovial fluid, is a relatively new concept. It has long been hypothesised specific entire bacteria may be present in synovial fluid and could be implicated in RA, although the underlying mechanism remains unclear (Zhao *et al.*, 2018a). A recent molecular study has indicated that there was bacterial dysbiosis of synovial fluid in RA patients (Zhao *et al.*, 2018a). However, no information was available regarding fungal dysbiosis in RA synovial fluid. Such alterations in the synovial fluid have the potential to increase our disease understanding and further suggest that the microbiome may afford a valuable source of novel biomarkers and or novel targets for therapeutic modulation (Zhao *et al.*, 2018a).

In this chapter, I characterised the synovial fluid microbiome of RA patients and compared those to healthy control subjects. This allowed the assessment of any apparent changes in the bacterial or fungal populations identified in the context of key markers of inflammation in synovial fluid (IL-6, IL-17A, IL-22, and IL-23). Here, I investigated the presence of bacterial (via sequencing of the 16S rRNA gene) and fungal (via sequencing of the ITS2 region) DNA in the synovial fluid of human RA patients and healthy control subjects using cutting-edge next-generation sequencing and bioinformatic techniques. Finally, I evaluated any apparent changes in the bacterial or fungal populations detected in the context of crucial markers of synovial inflammation (IL-6, IL-17A, IL-22, and IL-23).

4.2 Methods

Chapter 4 provides the methods for 16S rRNA and ITS2 PCR step, Gel electrophoresis, DNA purification by Qiagen Purification Kit, Addition of Illumina XT tags, and DNA purification by AMPure XP magnetic beads. It also gave methods for DNA sequencing utilising an Illumina MiSeq and subsequent bioinformatic analysis and further assessment of the IL-6, IL-17A, IL22, and IL-23 concentration in synovial fluid **(See Methods section).**

4.3 Results

4.3.1 Clinical characteristics of cohorts and results of 16S rRNA and ITS2 PCR amplification

Synovial fluid samples were obtained from 25 human donors. Of these, sixteen patients were diagnosed with rheumatoid arthritis, including eight males and eight females. The RA patients' ages ranged from 52 to 74 years, with a mean of 65.3 years. Nine control synovial fluid samples were obtained from 5 males and 4 females. Their ages ranged from 50 to 68 years with a mean 61 years. There were no significant differences in the ages of the two donors (Unpaired T-test; P = > 0.05**).**

The utilisation of the PCR amplification showed that bacterial 16S DNA was detected in the synovial fluid of 14 out of 16 patients with RA (87.5%), and in 9 out of 9 (100%) healthy control subjects. ITS2 amplification, indicative of the presence of fungi, was detected in 12 of 16 (75%) RA samples and 8 out of 9 (89%) healthy control samples, as shown in **Table**

4-1. Our various experimental negative controls of PCR experiments/ kit controls of purification step by PCR Purification Kit) constantly did not generate a visible band after PCR and agarose gel electrophoresis. In addition, DNA quantification utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen) confirmed this non-appearance. The Qubit 3.0 high-sensitivity DNA kit is designed to be accurate for initial sample concentrations from 10 pg/μl; in the case of our negative control samples, zero values were obtained.

Table 4-1 Features of RA patients and healthy population are taken from Sera Lab Company, and results of 16S rRNA and ITS2 PCR

Patient ID#	Gender	Age	Diagnosis	16S rRNA PCR	ITS2 PCR
1339	Female	65	RA	+	−
1340	Female	67	RA	+	+
1341	Female	67	RA	+	+
1342	Female	67	RA	+	−
1343	Female	70	RA	+	−
1344	Female	69	RA	+	−
1345	Male	56	RA	+	+
1346	Male	52	RA	+	+
1347	Male	55	RA	+	+
1348	Male	66	RA	+	+
1349	Male	74	RA	+	+
1350	Male	69	RA	+	+
BRH1095336	Female	67	RA	+	+
BRH1095337	Female	67	RA	−	+
BRH1095338	Male	67	RA	−	+
BRH1095339	Male	67	RA	+	+
1351	Female	57	Healthy	+	+
1352	Male	64	Healthy	+	−
1353	Male	64	Healthy	+	+
1354	Male	53	Healthy	+	+
1355	Male	50	Healthy	+	+
1356	Male	74	Healthy	+	+
1357	Female	64	Healthy	+	+
1358	Female	55	Healthy	+	+
1359	Female	68	Healthy	+	+

4.3.2 **Estimation of inflammatory cytokines in synovial fluid**

Synovial fluid levels of interleukin 6 (IL-6), 17A (IL-17A), 22 (IL-22), and 23 (IL-23) were measured utilising the Luminex system as described in the methods and materials section in chapter 2. Mean interleukin concentrations in the synovial fluid of healthy control subjects and RA patients were significantly different, with cytokines present at higher levels in the RA synovial fluid in all cases **(Figure 4-1)**.

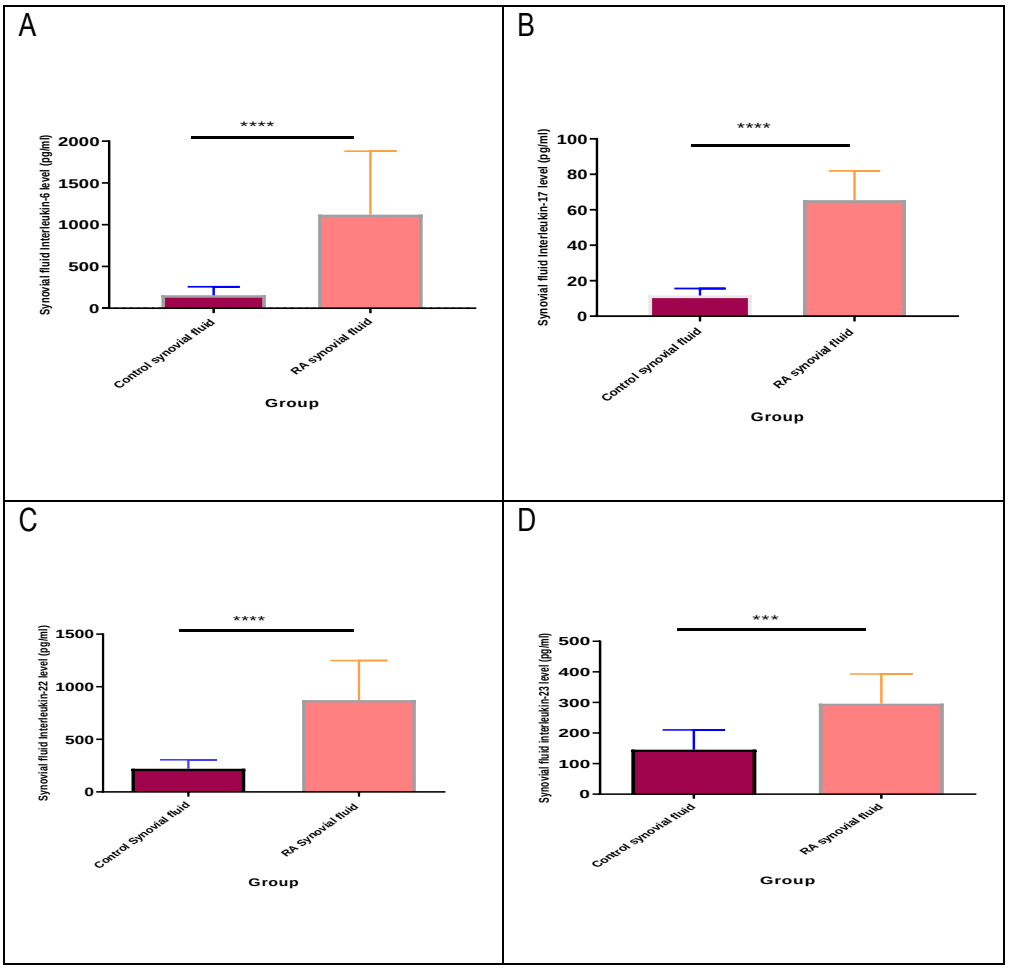


Figure 4-1 Human Magnetic Luminex Screening Assay measured IL-6, IL-17, IL-22, and IL-23 levels in synovial fluid (SF) from RA patients and healthy controls. IL-6, IL-17, IL-22, and IL-23 levels were increased in the synovial fluid of RA patients relative to the synovial fluid of healthy controls. Values represent the mean+SD of cytokine levels (pg/ml). The statistical significance between groups was determined by unpaired T-test. ***P < 0.001, ****P < 0.0001.

4.3.3 Characterisation of Bacterial populations via 16S rRNA sequencing of synovial fluid

The existence of bacterial DNA in synovial fluid was evaluated via PCR amplification and sequencing of the bacterial 16S rRNA gene, variable region 4. An average of 75,000 reads was produced for each of the samples (70,658 reads for RA, 80,533 reads for Control). Although the control samples produced more reads on average, this difference was not statistically significant (P = > 0.05). Principal coordinates analysis (PCoA) was carried out to reduce the complexity of the data obtained and to visualise any differences in bacterial structure between the two cohorts **(Figure 4-2).** There is a wide spread of microbiome variation across the RA synovial fluid samples.

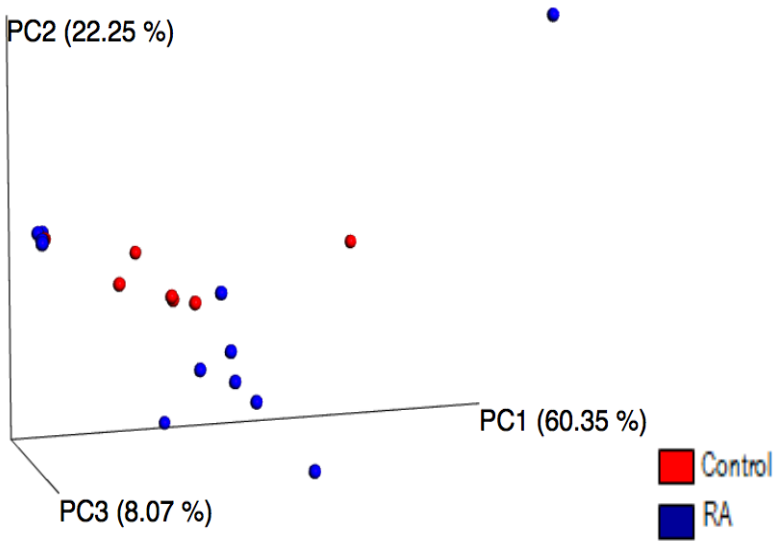


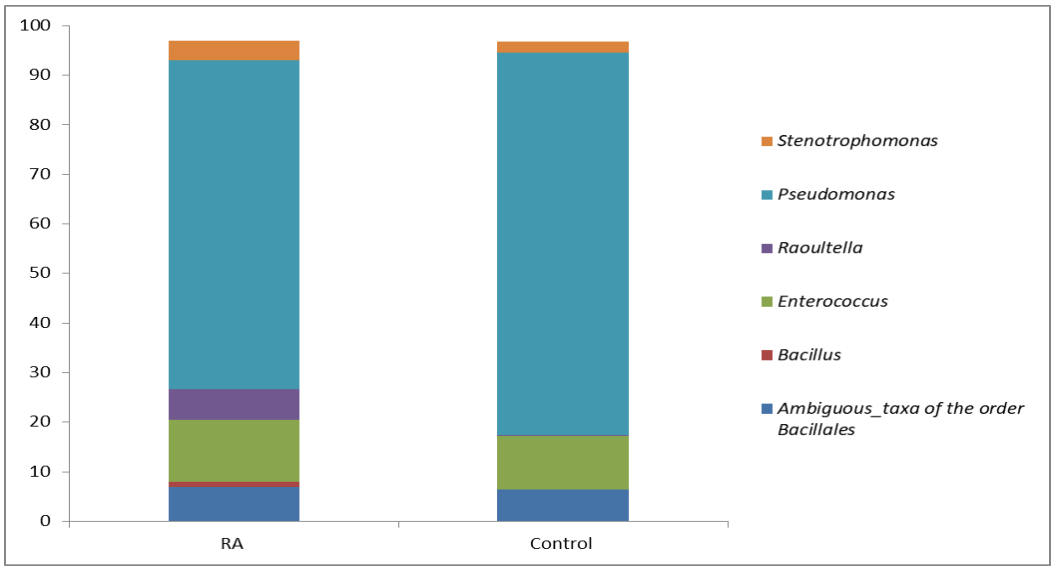
Figure 4-2 Principal Coordinate Analysis plot generated through a Bray Curtis distance matrix of bacterial 16S rRNA synovial fluid for control subjects (red) and arthritic patients (blue) as determined via amplification and sequencing of 16S rRNA gene variable region 4 (V4). Proportions of variation explained by the principal coordinates are designated on the axes. PCoA identified that the maximal variation was 60.35% (PC1), 22.25% (PC2) and, 8.07% (PC3). The microbiome of samples that appear in close proximity to each other is more similar in composition. There is also a wide spread of microbiome variation across the RA synovial fluid samples.

The results are shown at the phylum and genus levels. At the phylum level, *Proteobacteria* (81%) and *Firmicutes* (16.5%) are dominant members, and to a much lesser extent,

Actinobacteria (0.3%) and *Bacteroidetes* (0.1%). Although the most important phyla identified are similar to those of the blood microbiome (Whittle *et al.*, 2019), they are existent in the synovial fluid at different levels, thus proposing a different community structure.

At the genus level, our synovial fluid samples were dominated by the genus *Pseudomonas* (RA= 66.1%, Control= 80.6%), followed by the genus *Enterococcus* (RA= 11.4%, control=11.7%). To a lesser extent, the synovial fluid samples contained taxa of the *Bacillales; Ambiguous_taxa; Ambiguous order* (RA=7.4%, control = 4%), and *Stenotrophomonas* genus (RA= 4.2%, control= 1.5%). Furthermore, the genera *Raoultella* (6.5%) and *Bacillus* (1.2%) were detected in RA synovial samples only **(Figure 4-3).** A Mann–Whitney U test identified the abundance of *Raoultella* as marginally significant; observed as an increase in RA synovial fluid samples in comparison to healthy populations **(Table 4-2 and Figure 4-4).**

A-



B-

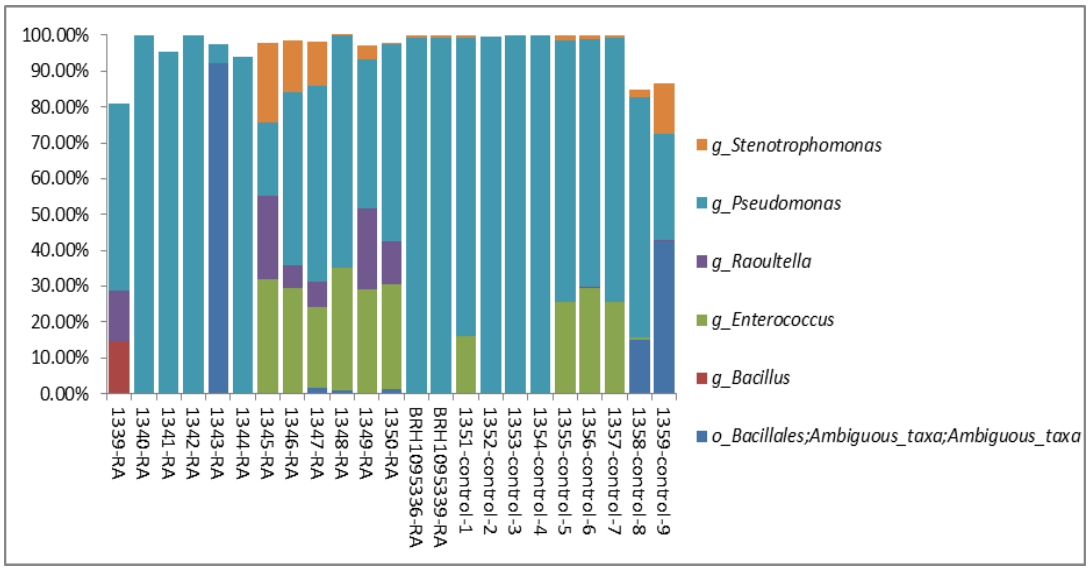


Figure 4-3 Relative abundance of abundant bacterial genera detected in human synovial fluid. Bars show the relative abundance of the common bacterial taxa, characterized as having a mean abundance of >1%, in synovial fluid samples of rheumatoid arthritis (RA, n = 14) and control (Control, n = 9) samples as determined by amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean abundance expressed as a percentage of the total bacterial sequence count. **A-** Taxa data grouped by condition and **B-** Taxa individual sample data.

Table 4-2 Statistical analysis of the relative abundances of common bacterial genera detected in the synovial fluid samples of healthy control and RA subjects, with expression levels >1%. Data are displayed as a median (SD). P values were determined by applying a two-tailed, Mann Whitney test using GraphPad Prism V8. P <= 0.05 was a statistically significant

Taxa	Control Median (SD)	RA Median (SD)	P value
Order			
Bacillales;Ambiguous_taxa;Ambiguous_taxa	0 (14.4)	0.05 (24.5)	0.97
Genus			
Stenotrophomonas	0.9 (4.5)	0.15 (7.1)	0.97
Pseudomonas	73.6 (22.3)	60 (32)	0.30
Raoultella	0 (0.1)	0 (8.7)	**0.051**
Enterococcus	0.6 (13)	0.1 (15.2)	0.95
Bacillus	0 (0)	0 (3.9)	0.99

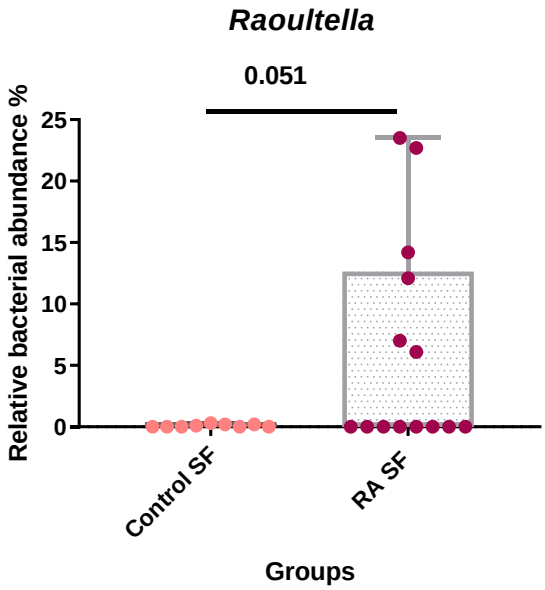


Figure 4-4 Relative abundance of the genus *Raoultella* identified in the synovial fluid of healthy donors and rheumatoid arthritis patients as determined by amplification and sequencing of the 16S rRNA gene variable region. There was a marginally significant ($p = 0.051$) increase in the abundance of *Raoultella* in the synovial fluid of rheumatoid arthritis patients relative to healthy subjects. In fact, only genus *Raoultella* was existent in RA synovial fluid, absent in healthy control synovial fluid. Data are median abundance expressed as a percentage of the total bacterial sequence count. Statistical significance was determined by a Mann–Whitney U test with $P < 0.05$ considered significant.

4.3.3.1 Synovial fluid microbiome of patients with RA examined across gender

We examined whether the relative abundance of specific bacterial taxa (>1%) might differ among males (n=7) and females (n=7) RA patients, which might explain microbiome variability in the RA patient population in PCoA **(Figure 4-2).**

PCoA was performed to reduce the complex, multidimensional data for visualisation of patterns and to assess whether the synovial fluid microbial community could be differentiated between men and women patients with RA. Following ordination, it was immediately apparent that the RA men samples clustered markedly further away from the women samples. These results propose that the bacterial population composition found in male RA patients' synovial fluid was more markedly different from that of women **(Figure 4-5).**

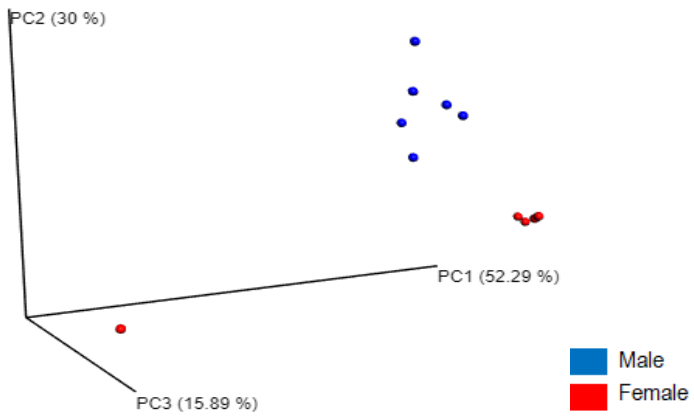


Figure 4-5 PCoA plot informed by weighted unifrac distance matrix for male (blue) and female (red) of patients with RA. Distance matrix was informed by amplification and sequencing of the 16S rRNA variable region 4, followed by a taxonomic assignment. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 52.29 % (PC1), 30 % (PC2) and, 15.89 % (PC3). PCoA shows a clear difference in the microbiome composition between men and women patients with RA.

A Mann–Whitney U test identified that the member of *Enterococcus* (P = 0.004), *Stenotrophomonas* (P = 0.002) genera were found almost exclusively in RA synovial fluid samples from men only compared to women. Further, the abundance of *Raoultella* as marginally significant; it was observed as an increase in males RA synovial fluid samples compared to females **(Figure 4-6).** These data suggested that differences exist between males and females synovial fluid microbiome populations of patients with RA.

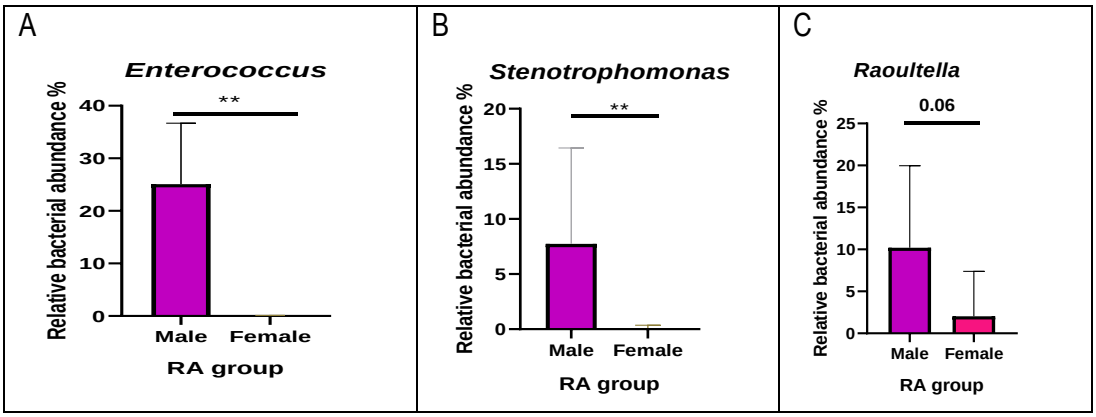


Figure 4-6 Relative abundance of *(A) Enterococcus, (B), Stenotrophomonas,* and (C) *Raoultella* identified in men and women synovial fluid microbiome populations of patients with RA. Data determined through the amplification and sequencing of the 16s rRNA V4. (A and B) The abundance of the *Enterococcus* and *Stenotrophomonas* have been detected almost exclusively in RA synovial fluid samples from males only in comparison to females. (C) A borderline significant (p = 0.06) increase in the abundance of genus *Raoultella* was found in RA synovial fluid of males relative to females. Values represent the mean (SD) of abundance expressed as a percentage of the total bacterial sequence count. Statistical significance was determined by a Mann–Whitney U test with P < 0.05 considered significant, **P < 0.01.

4.3.3.2 Synovial fluid microbiome of patients with RA examined across age

We next determined whether synovial fluid microbiome, represented by the bacteria, differed between aged ≤ 65 years, which are ranged age group from 52-65, n=4 and aged >65 years which are ranged age group from 66-74 years old, n=10 of RA patients populations, which may explain the microbiome variability in the RA population. PCoA was carried out to reduce the complex, multidimensional data for visualisation of microbiome patterns and to assess whether the synovial fluid microbial community could be differentiated between RA patients age ≤ 65 years and > 65 years **(Figure 4-7).**

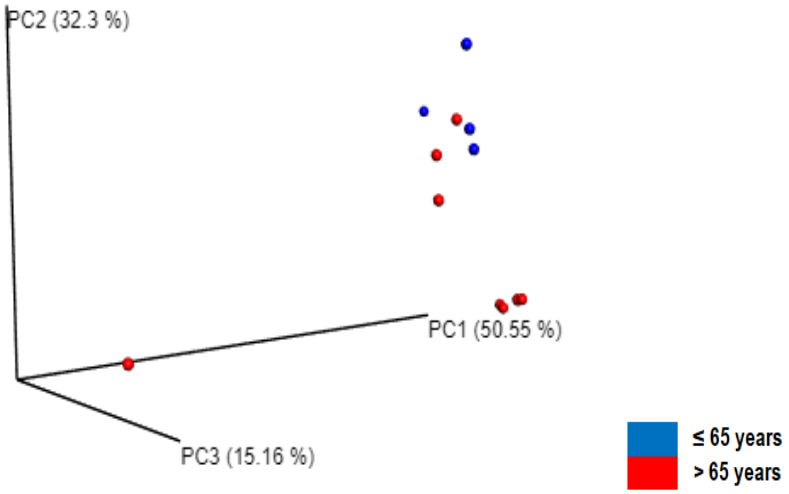


Figure 4-7 PCoA plot informed by weighted unifrac distance matrix for ≤ 65 (blue) and >65 years (red) of patients with RA. Distance matrix informed by amplification and sequencing of the 16S rRNA variable region 4, followed by a taxonomic assignment. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 50.55 % (PC1), 32.3 % (PC2) and, 15.16 % (PC3).

A Mann–Whitney U test revealed that an abundance of *Raoultella* was significantly increased in RA patients with age ≤ 65 compared to RA patients with >65 **(Figure 4-8).**

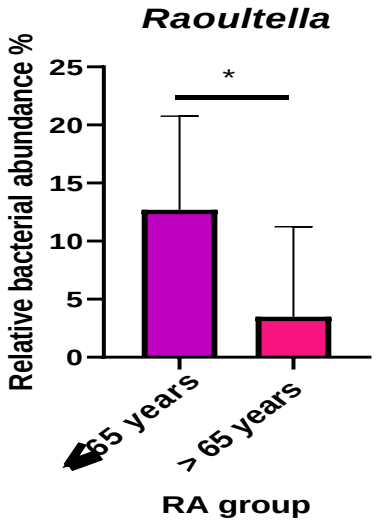


Figure 4-8 Relative abundance of the genus *Raoultella* found in the synovial fluid with age ≤ 65 and >65 of RA patients as determined by amplification and sequencing of the 16S rRNA gene variable region. There was a significant increase in the abundance of *Raoultella* in RA synovial fluid with age ≤ 65 relative to age >65. Data are mean (SD) abundance expressed as a percentage of the total bacterial sequence count. Statistical significance was determined by a Mann–Whitney U test with $P < 0.05$ considered significant.

4.3.3.3 The effect of age and gender on *Raoultella* genus in RA patients

Analysis of the effect of gender and age on *Raoultella* abundance in patients with RA revealed that there was a borderline significant ($p = 0.06$, Mann Whitney test) increase in the abundance of genus *Raoultella* in RA synovial fluid of males in comparison to females **(Figure 4-6.C).** Further, an abundance of *Raoultella* was significantly ($p \leq 0.05$, Mann Whitney test) increased in RA patients with age ≤ 65 compared to RA patients with >65 **(Figure 4-8).** These findings showed that increased *Raoultella* abundance of RA patients could be associated with male's gender and with age ≤ 65.

4.3.3.4 Altered inflammatory cytokine responses in RA patients with alterations in taxonomic compositions of the *Raoultella*

To investigate whether the presence of *Raoutella* in RA patients impacts on the inflammatory cytokines (IL-6, IL-17, IL-22, IL-23), an unpaired T-test was applied. An unpaired T-test indicates that there was no significant statistical difference in the production of inflammatory cytokines between RA patients with the presence of Raoutella and other RA patients with the absence of *Raoutella* **(Table 4-3).** This indicates that the concentrations of inflammatory cytokines were not affected by the presence of *Raoutella* in our RA patients.

Table 4-3 Comparison of IL-6, IL-17, IL-22, and IL-23 cytokine concentrations in RA patients samples, in which *Raoultella* presence and with RA patients samples, in which *Raoultella* absence. The data are represented as the mean (SD) **of cytokine levels (pg/ml). The statistical significance between groups was determined by unpaired T-test.**

Inflammatory cytokines	RA patients with Raoutella Mean(SD) mg/dl	RA patients without Raoutella mean(SD) mg/dl	P-Value
IL-6	1061(331)	1755(1314)	ns
IL-17	62.5(16)	65.7(19.3)	ns
IL-22	898(367)	925(325)	ns
IL-23	344(77)	292(94)	ns

We next examined whether there are specific inflammatory cytokine responses associated with the relative abundances of *Raoutella* in RA patients. *Spearman's* rank correlation coefficient test found that the correlation was not observed between the concentrations of inflammatory cytokines and the abundance of *Raoutella* **(Table 4-4).**

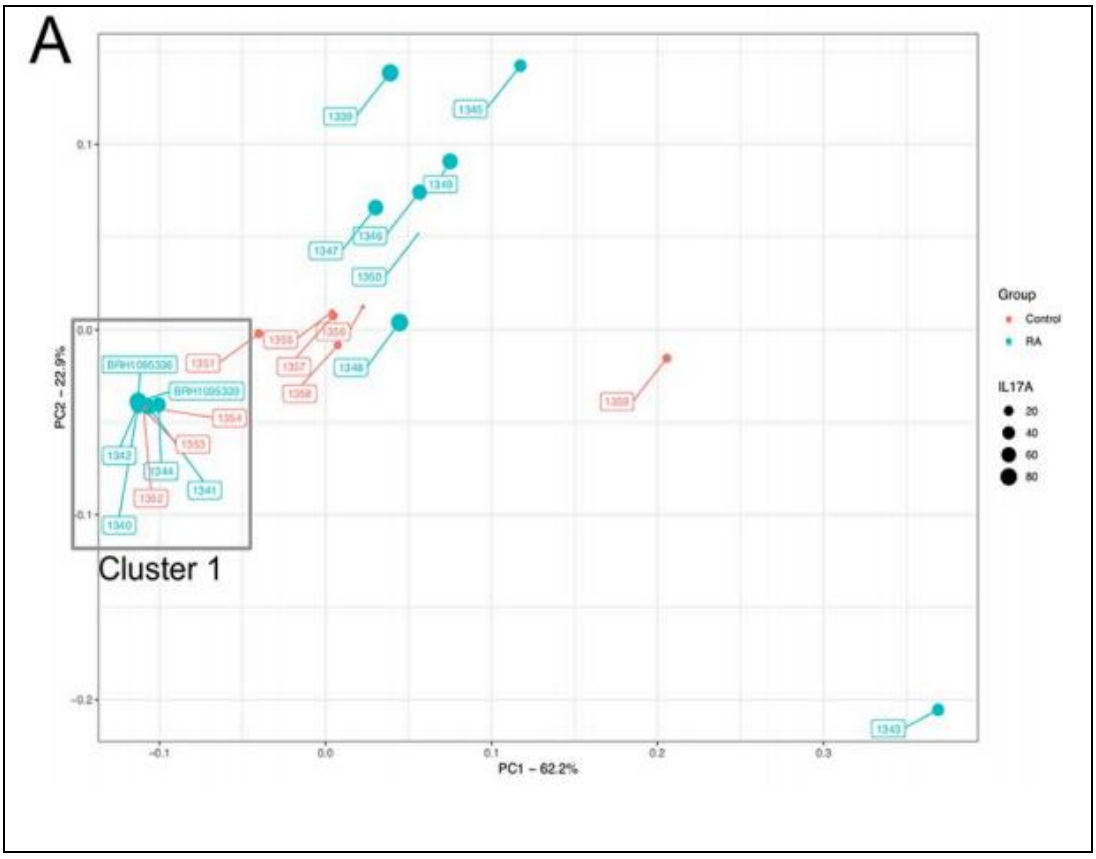
Table 4-4: Spearman's rank correlation determines the correlation of synovial fluid *Raoultella* genus, which is significantly changed via RA status with inflammatory cytokines (IL-6, IL-17, IL-22, and IL-23).

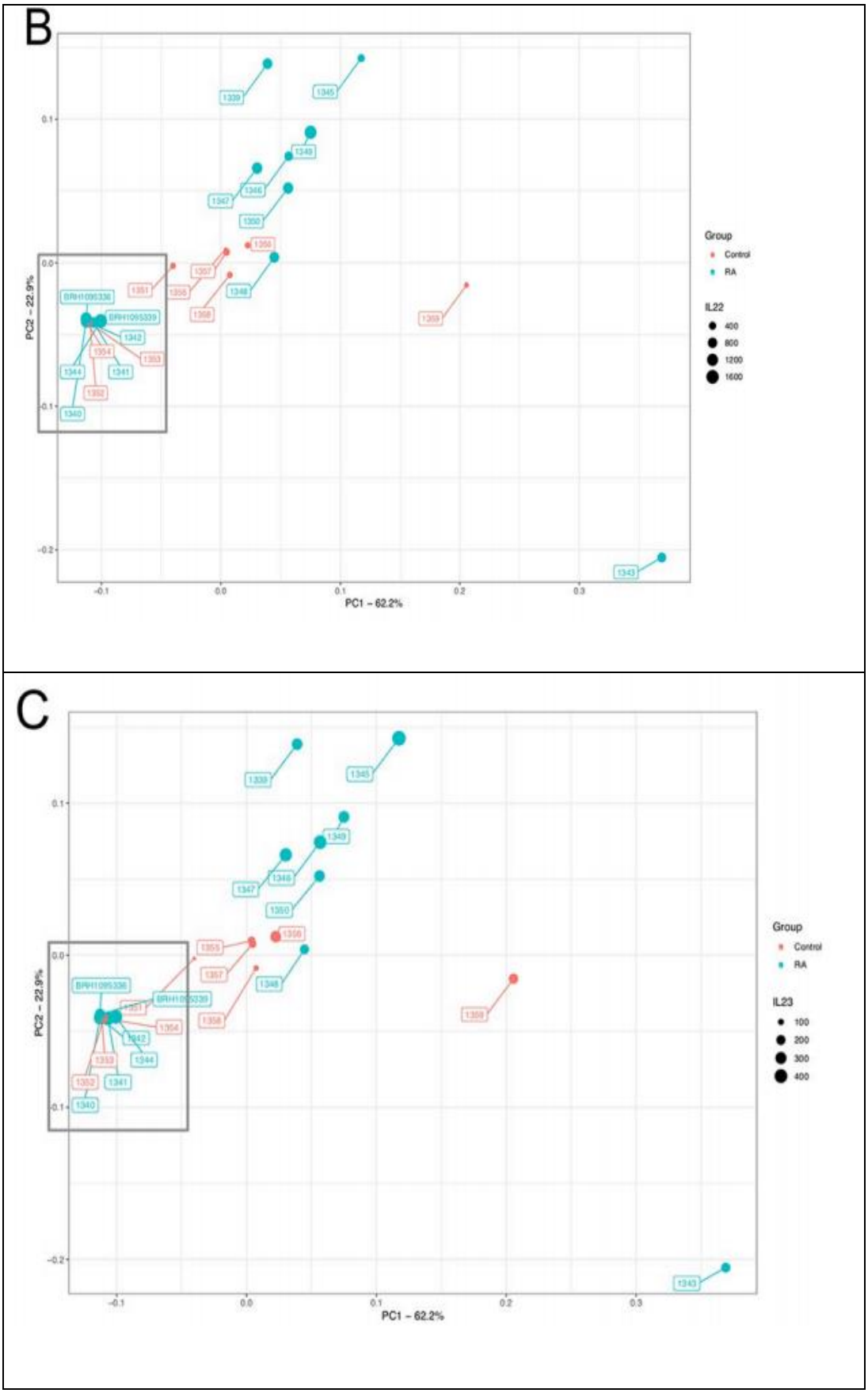
Raoultella taxa vs. cytokine	Spearman's rank correlation	P value
Raoultella vs. IL-6	-0.13	ns
Raoultella vs. IL-17	-0.08	ns
Raoultella vs. IL-22	-0.05	ns
Raoultella vs. IL-23	0.2	ns

4.3.4 **Inflammatory markers are modulated by bacterial microbiome PCoA cluster**

Based on the clustering of samples followed by PCoA **(Figure 4-9),** we examined whether the samples in "cluster 1" of RA patients had significantly different levels of inflammatory markers from the remaining samples as well as different bacterial communities.

The important point here, IL-6 was a higher in PCoA cluster 1 **(RA cluster 1 mean (SD) = 2118 (1320) pg/ml)** in comparison with the remaining samples **(RA cluster 2 mean (SD) = 962.4 (357.8) pg/ml), (**unpaired T-test; P = 0.0348). However, all other levels of inflammatory markers were unchanged by PCoA cluster (P > = 0.05) **(Figure 4-10).**




B
C
PC1 – 62.2%
PC2 – 22.9%
Group
Control
RA
IL22
400
800
1200
1600
IL23
100
200
300
400

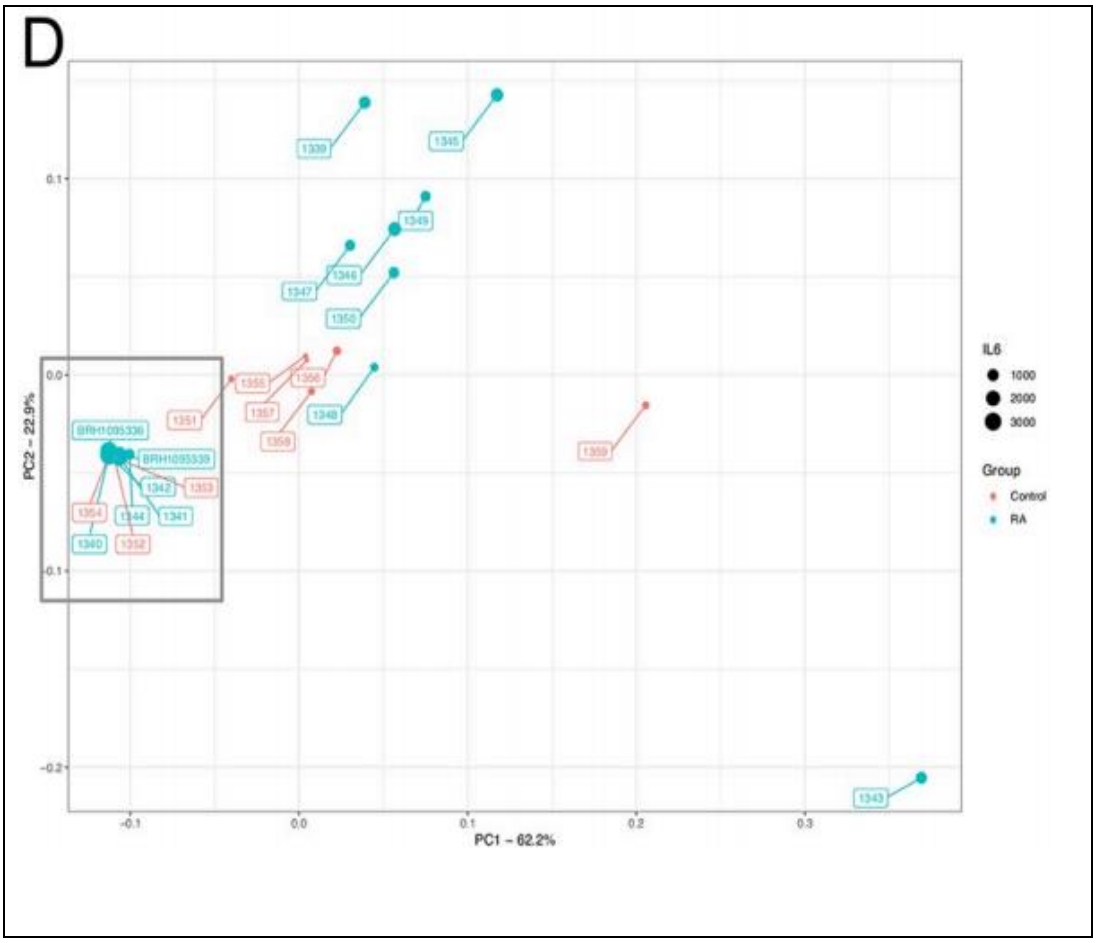


Figure 4-9 Principal Coordinate Analysis plot generated from a Bray Curtis distance matrix of synovial fluid bacterial community structure for control subjects (red) and arthritic patients (blue), whereas the data points in each pane are sized according to the synovial level of IL-17A (A), IL-22 (B), IL-23 (C), and IL-6 (D). Data determined via amplification and sequencing of the 16S rRNA gene variable region 4 (V4). Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 62.2% (PC1), and 22.9% (PC2). The microbiome of samples that appear in close proximity to each other is more similar in composition.

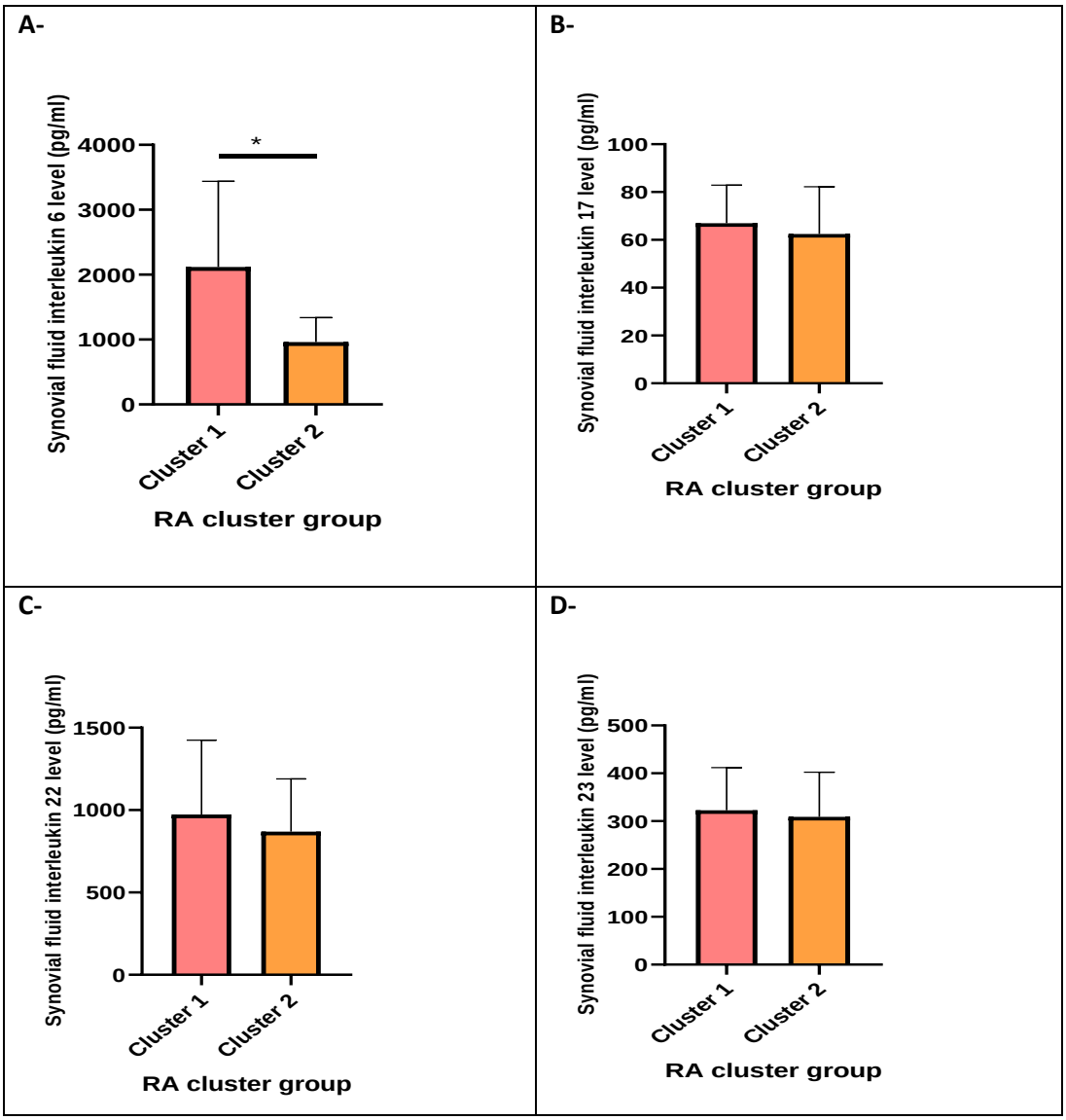


Figure 4-10 Human Magnetic Luminex Screening Assay measured IL-6, IL-17, IL-22, and IL-23 levels in the synovial fluid of the bacterial RA clusters (cluster1 vs. cluster2). A, the level of IL-6 was a significant increase in RA cluster1 relative to cluster2. **B-D,** IL-17, IL-22, and IL-23 levels were not significantly changed between RA cluster1 and cluster2. Values represent the mean+SD of cytokine levels (pg/ml). The statistical significance between groups was determined by unpaired T-test, *P < 0.05.

4.3.5 **Characterisation of fungal populations via ITS2 sequencing of synovial fluid**

The existence of fungal DNA in synovial fluid was evaluated via PCR amplification and sequencing of the fungal ITS2 gene. Averages of 50,000 reads were produced for each of the samples (52,113 reads for RA, 49,929 reads for Control). Although the RA samples produced more reads on average, this difference was not statistically significant (P = > 0.05). Principal coordinates analysis (PCoA) was performed to show the separation between the groups based upon their fungal population's profile **(Figure 4-11).**

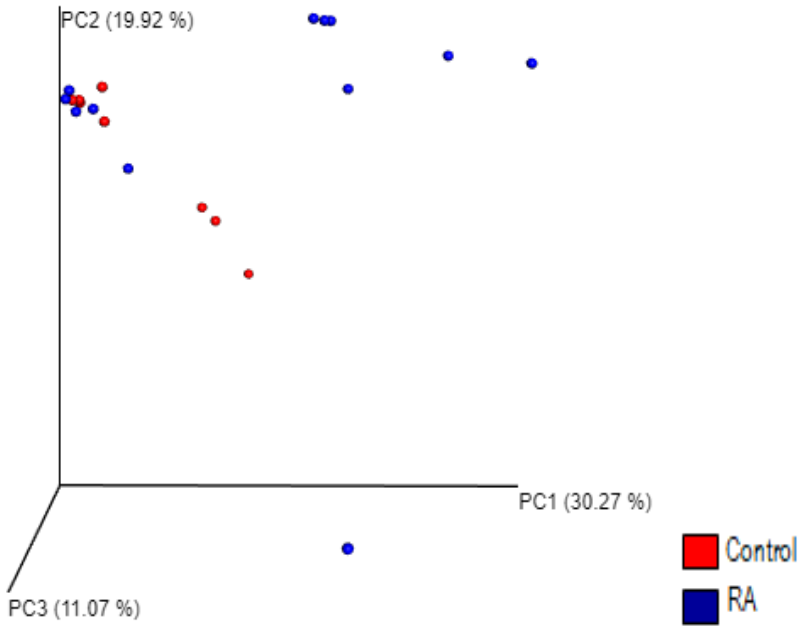
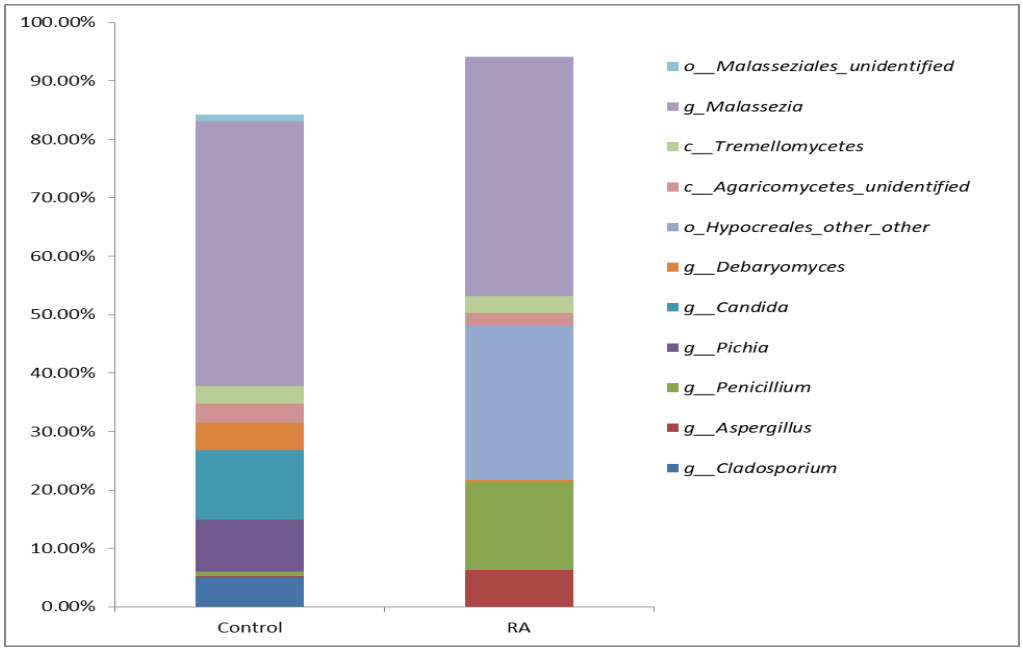


Figure 4-11 Principal Coordinate Analysis plot generated a Bray Curtis distance matrix of fungal ITS2 synovial fluid for control subjects (red), and arthritic patients (blue) as determined via amplification and sequencing of ITS2 Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 30.27% (PC1), 19.92% (PC2) and, 11.07% (PC3). The microbiome of samples that appear in close proximity to each other is more similar in composition.

At the phylum level, synovial fluid was identified to be dominated by members of the *Basidiomycota* (50.4%) and *Ascomycota* (35.1%) phyla. At the genus level, our synovial fluid samples were predominated by the genus *Malassezia,* which accounted for 40.9% and 45.3% of the total bacterial sequence identified in the RA and controls donors, respectively. To a lesser extent, the synovial fluid samples also contained unclassified organism belonging to the classes *Tremellomycetes* (2.8%, 3%), *Agaricomycetes* (2.2%, 3.2%), and genus *Penicillium* (0.7%, 15.2%). Moreover, members of the *Hypocreales* order (26.4%), and the genus *Aspergillus* (6.2%) were detected in RA synovial samples only. However, unclassified organisms belonging to the order *Malasseziales* (1.2%), genus Candida (11.9%), *Pichia* (8.9%), *Cladosporium* (5.1%), and *debaryomyces* (4.7%) were observed in control healthy subjects **(Figure 4-12).**

A Mann–Whitney U test found that while the vast majority of the fungal population was unaltered via illness, select fungal taxa were differentially abundant or existent **(Table 4-5 and Figure 4-13).** The member of the *Hypocreales* order ($P = 0.012$) and a member of the genus *Aspergillus* was identified almost exclusively in RA synovial fluid samples only ($P = 0.14$ including all data, $P = 0.057$ excluding samples where <1% of reads mapped to this taxa). However, unclassified organisms belonging to the order *Malasseziales* ($P = 0.002$) and genus *Cladosporium* ($P = 0.019$) were found in control synovial fluid only.

A-



B-

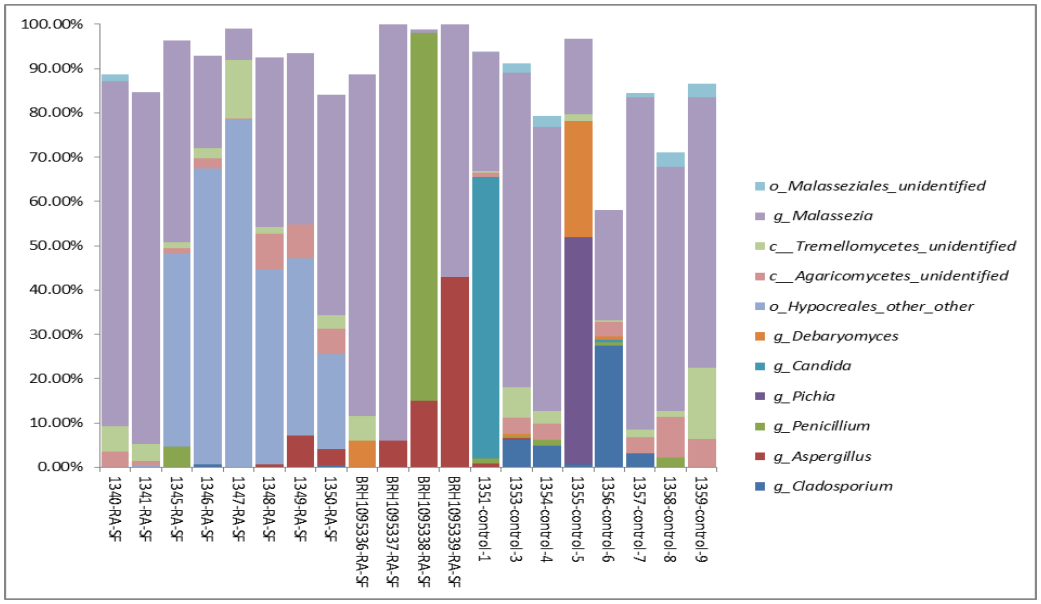


Figure 4-12 Relative abundance of abundant fungal genera in synovial fluid. The Bar shows the relative abundance of the common fungal taxa, characterised as having a mean abundance of >1%, in synovial fluid samples of rheumatoid arthritis (RA, n = 12) and Control (Control, n = 8) samples as determined by amplification and sequencing of the fungal ITS2 gene. Data are mean abundance expressed as a percentage of the total bacterial sequence. **A-** Taxa data grouped by condition and **B-** Taxa individual sample data.

Table 4-5 Statistical analysis of the relative abundances of common fungal genera detected in the synovial fluid samples of healthy control and RA subjects, with expression levels >1%. Data are a median (SD). P values were determined by applying a two-tailed, Mann Whitney test using GraphPad Prism V8, P <= 0.05 was considered statistically significant.

Taxa	Control abundance median (SD)	RA abundance median (SD)	P value
Class			
Tremellomycetes _unidentified	1.6 (5.3)	1.9 (3.8)	0.98
Agaricomycetes _unidentified	3.6 (2.8)	1 (3)	0.3
Order			
Hypocreales_other_other	0 (0)	11 (24.6)	**0.012**
Malasseziales _unidentified	1.6 (1.3)	0 (0.4)	**0.002**
Genus			
Aspergillus	0 (0.3)	0.3 (12.4)	**0.057**
Cladosporium	1.9 (9.3)	0 (0.1)	**0.019**
Malassezia	58 (23)	47.7 (30)	0.8
Penicillium	0.6 (0.8)	0 (24)	0.6
Pichia	0 (6.4)	0 (0)	0.4
Debaryomyces	0 (9.2)	0 (1.7)	0.7
Candida	0 (22.4)	0 (0)	0.4

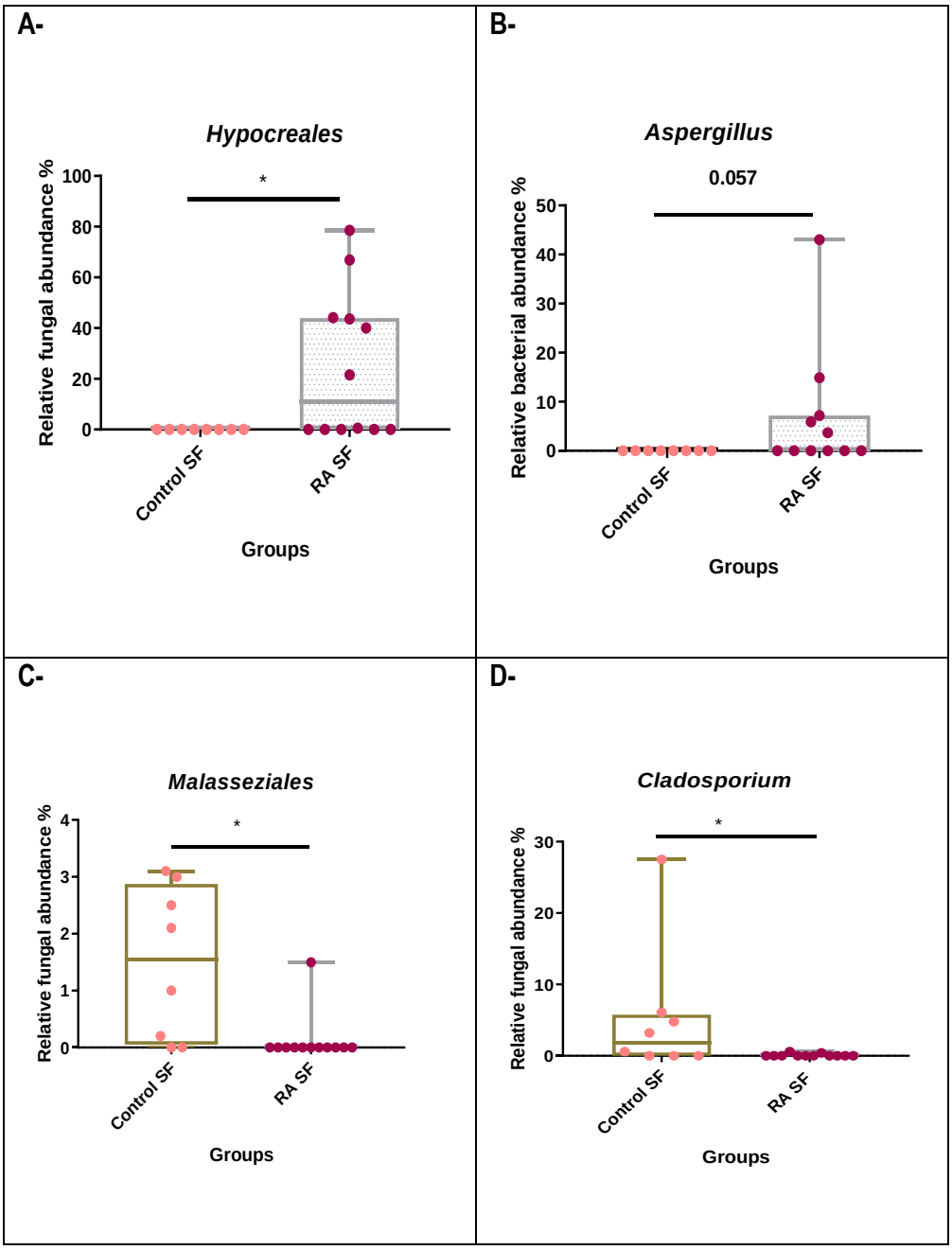


Figure 4-13 Relative abundance of *(A) Hypocreales, (B), Aspergillus, (C) Malasseziales*, and (D) *Cladosporium* found in the synovial fluid of rheumatoid arthritis patients and healthy control subjects. Data determined through the amplification and sequencing of the ITS2 gene. (A) The relative abundance of order *Hypocreales* was increased in the synovial fluid of RA patients compared with healthy control synovial fluid. (B) A borderline significant ($p = 0.057$) increase in the abundance of genus *Aspergillus* was identified in RA synovial fluid relative to healthy control synovial fluid. (C), and (D), The abundance of the unclassified organism belonging to the order *Malasseziales* and genus *Cladosporium* were significantly decreased in the synovial fluid of RA patients in comparison to the synovial fluid of healthy control subjects. Data are individual expression values expressed as a percentage of the total fungal read count in each sample. The statistical significance between groups was determined by a Mann–Whitney U test where $P \leq 0.05$.

4.3.5.1 The effect of age and gender on *Hypocreales* taxa in RA patients

Analysis of the effect of gender and age on *Hypocreales* abundance in patients with RA identified that there was a borderline significant (p = 0.06, Mann Whitney test) increase in the abundance of *Hypocreales* in RA synovial fluid of males when compared those with females **(Figure 4-14).**

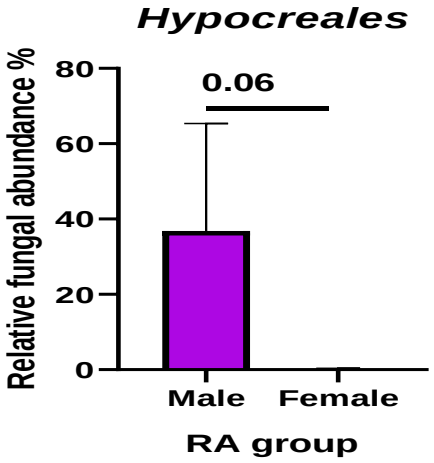


Figure 4-14 Relative abundance of *Hypocreales* found in men and women synovial fluid microbiome populations of patients with RA. Data determined through the amplification and sequencing of the ITS2. A borderline significant (p = 0.06) increase in the abundance of *Hypocreales* was identified in RA synovial fluid of males relative to females. The data are represented as the mean (SD) of abundance expressed as a percentage of the total fungal sequence count. Statistical significance was determined by a Mann–Whitney U test with P < 0.05 considered significant.

Further, an abundance of *Hypocreales* was significantly (p≤ 0.01, Mann Whitney test) increased in RA patients with age ≤ 65 compared to RA patients with >65 **(Figure 4-15).** These findings showed that increased *Hypocreales* abundance of RA patients could be associated with male's gender and with age ≤ 65.

Our results also showed that 5 out of 6 (83.3%) RA patients had elevated *Hypocreales* are the exact same patients identified with high *Raoutella*. Further, these patients are associated with the male gender. Moreover, there was a strong positive correlation between *Hypocreales* and *Raoutella* taxa in the synovial fluid of patients with RA **(Spearman's rank correlation = 0.7, P = 0.04) (Figure 4-16).**

These findings suggested that the male gender with RA is more susceptible to bacterial and fungal dysbiosis together, but not in the female. Therefore, the increase in *Raoultella* and *Hypocreales* in the RA male's synovial fluid of the same patients could be a biomarker to diagnose better, monitor, and treat this group of arthritic patients.

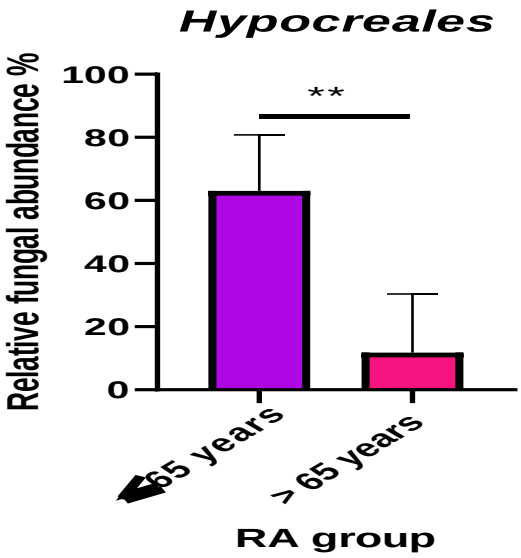


Figure 4-15 Relative abundance of the *Hypocreales* taxa observed in the synovial fluid with age ≤ 65 and >65 of RA patients as determined by amplification and sequencing of the ITS2 gene. There was a significant increase in the abundance of *Hypocreales* in RA synovial fluid with age ≤ 65 compared to age >65. Data are mean (SD) abundance expressed as a percentage of the total fungal sequence count. A Mann–Whitney U test determined statistical significance, **P < 0.01.

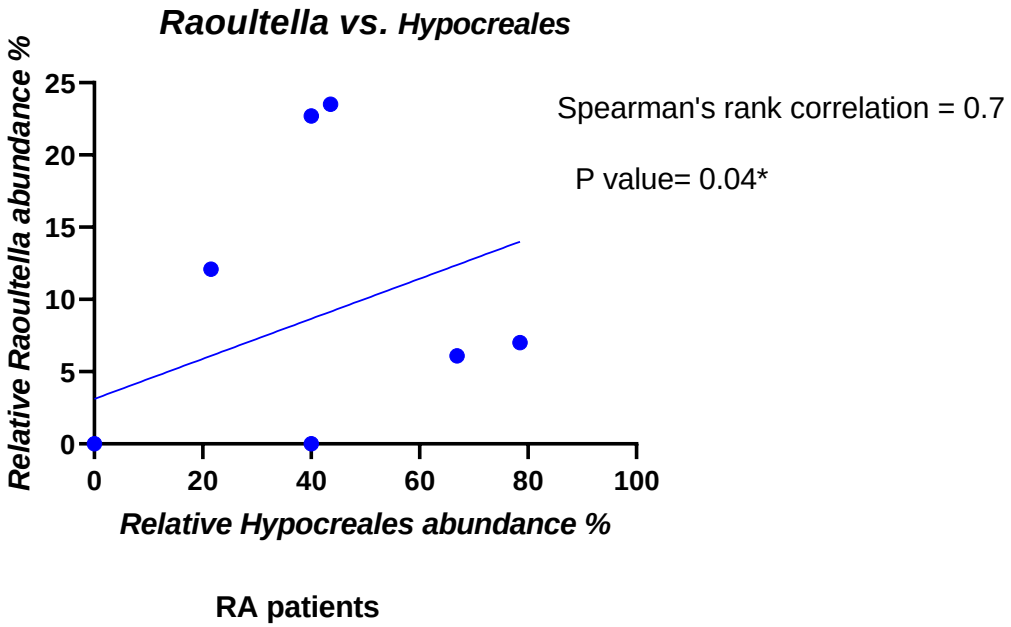


Figure 4-16 Correlation between the relative abundance of *Raoutella* and *Hypocreales* taxa identified in the synovial fluid of patients with RA. There was a strong positive correlation between the relative abundance of *Raoutella* and *Hypocreales* taxa detected in the synovial fluid of patients with RA. Data were statistically analysed using the Spearman correlation test. The level of statistical significance was set at a 95% confidence interval ($p < 0.05$), and the statistical analysis was determined using Prism 8.0 software. *$P < 0.05$.

4.3.5.2 Altered inflammatory cytokine responses in RA patients with alterations in taxonomic compositions of the *Hypocreales*

To examine whether the presence of *Hypocreales* in RA patients impacts on the inflammatory cytokines (IL-6, IL-17, IL-22, IL-23), an unpaired T-test was applied **(Table 4-6).** An unpaired T-test indicates that there was no significant statistical difference in the production of inflammatory cytokines between RA patients with the presence of *Hypocreales* and other RA patients with the absence of *Hypocreales*. This indicates that the concentrations of inflammatory cytokines were not affected by the presence of *Hypocreales* in our RA patients.

Table 4-6: Comparison of IL-6, IL-17, IL-22, and IL-23 cytokine concentrations in RA patients samples, in which *Hypocreales* presence and with RA patients samples, in which *Hypocreales* absence. The data are represented as the mean (SD) **of cytokine levels (pg/ml). The statistical significance between groups was determined by unpaired T-test.**

Inflammatory cytokines	RA patients with Hypocreales Mean(SD) mg/dl	RA patients without Hypocreales Mean(SD) mg/dl	P-Value
IL-6	1304(1065)	1851(1239)	ns
IL-17	64.7(16)	73.5(13)	ns
IL-22	893(336)	680(373)	ns
IL-23	342(91)	228(93)	ns

We next examined whether there are specific inflammatory cytokine responses associated with the relative abundances of *Hypocreales* in RA patients. *Spearman's* rank correlation coefficient test found that the correlation was not observed between the concentrations of inflammatory cytokines and the abundance of *Hypocreales* ***(Table 4-7).***

Table 4-7: Spearman's rank correlation determines the correlation of synovial fluid *Hypocreales*, which is significantly changed via RA status with inflammatory cytokines (IL-6, IL-17, IL-22, and IL-23).

***Hypocreales* taxa vs. cytokine**	**Spearman's rank correlation**	**P value**
***Hypocreales* vs. IL-6**	-0.3	ns
***Hypocreales* vs. IL-17**	-0.3	ns
***Hypocreales* vs. IL-22**	0.3	ns
***Hypocreales* vs. IL-23**	0.5	ns

4.3.6 Inflammatory markers are modulated by fungal microbiome PCoA cluster

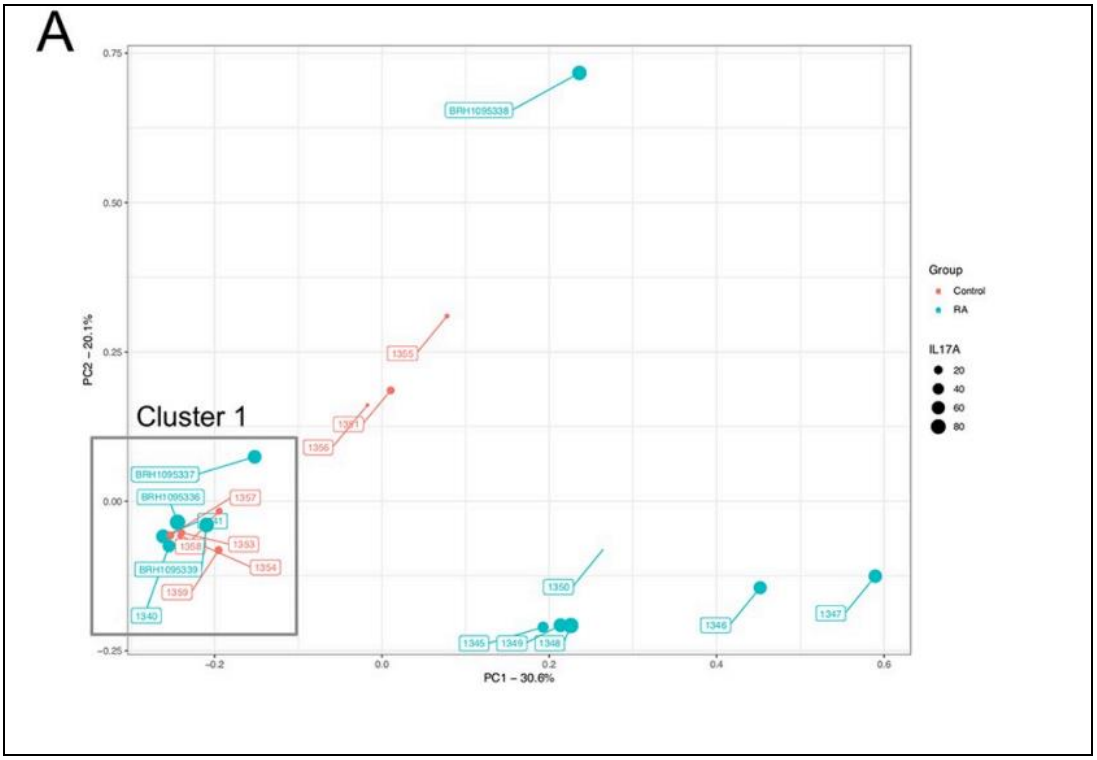
Based solely upon the clustering of samples observed following PCoA **(Figure 4-17),** we investigated whether samples in PCoA "cluster 1" of RA patients had significantly different inflammatory marker profiles, in addition to different fungal microbial populations.

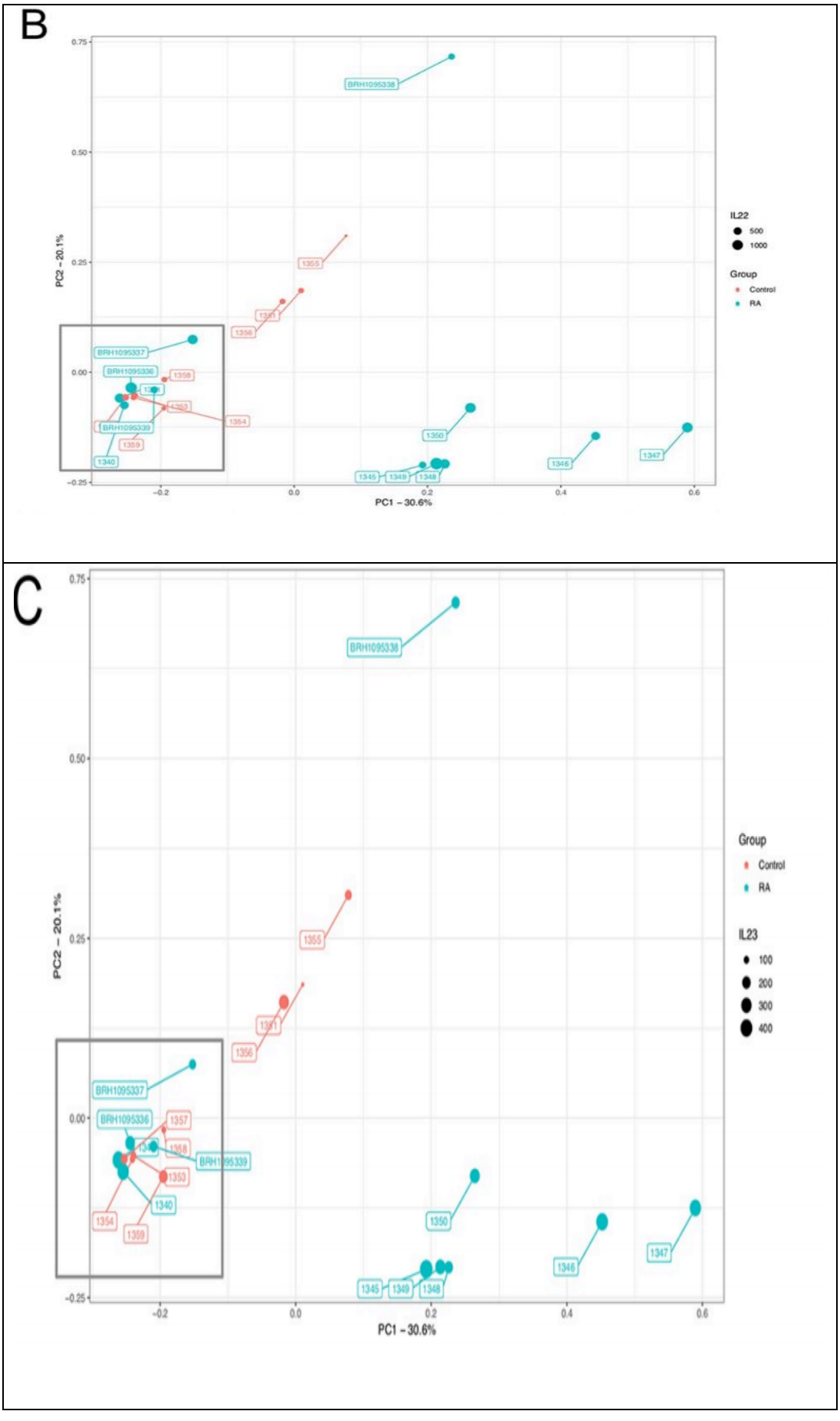
Interestingly, IL-6 was a higher in RA cluster 1 **(RA cluster 1 mean (SD) = 2362 (1318) pg/ml)** in comparison with the remaining samples **(RA cluster 2 mean (SD) = 939 (399.6) pg/ml),** (unpaired T- test; P = 0.02) **(Figure 4-18),** However, all other levels of inflammatory markers were unchanged by PCoA cluster (P > = 0.05).

Our observations also revealed that RA patients in cluster 1, **Figure 4-16,** are the same patients in cluster 1, **Figure 4-9** (the patients in Cluster 1 are the same patients in both the bacterial and fungal microbiome papulations). In addition, RA patients in the boxed cluster 1 are associated with female gender and age group with > 65 (aged 66–74).

This indicates that RA samples in “cluster 1” for bacterial and fungal populations had significantly inflammatory marker level, which is IL-6, to the remaining RA samples. Moreover, RA microbiome cluster 1 is related to particular characteristics, such as the female gender and age group with > 65.

Further, the patients in the boxed cluster 1 are the different patients, as in the patients discussed above who show differences in **Figures 4.4 & 4.13.**




B
PC2 – 20.1%
PC1 – 30.6%
IL22
500
1000
Group
Control
RA
BRH1095338
BRH1095337
BRH1095336
BRH1095339
1355
1356
1358
1363
1364
1359
1340
1350
1345
1349
1348
1346
1347
C
PC2 – 20.1%
PC1 – 30.6%
Group
Control
RA
IL23
100
200
300
400
BRH1095338
BRH1095337
BRH1095336
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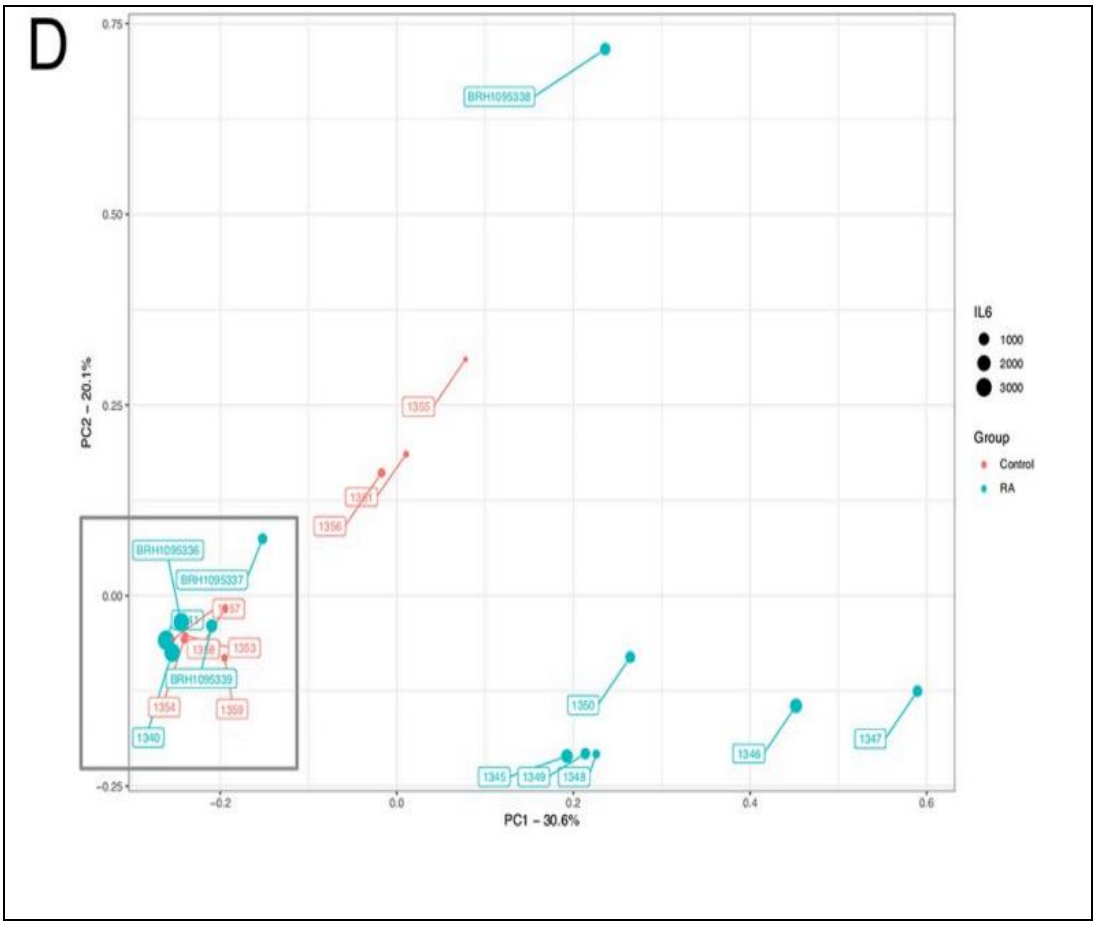


Figure 4-17 Principal Coordinate Analysis plot generated from a Bray Curtis distance matrix of synovial fluid fungal community structure for control subjects (red) and arthritic patients(blue), whereas the data points in each pane are sized according to the synovial concentration of IL-17A (A), IL-22 (B), IL-23 (C), and IL-6 (D). Data determined by amplification and sequencing of the ITS2 gene. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 30.6% (PC1), and 20.1%. The microbiome of samples that appear in close proximity to each other is more similar in composition.

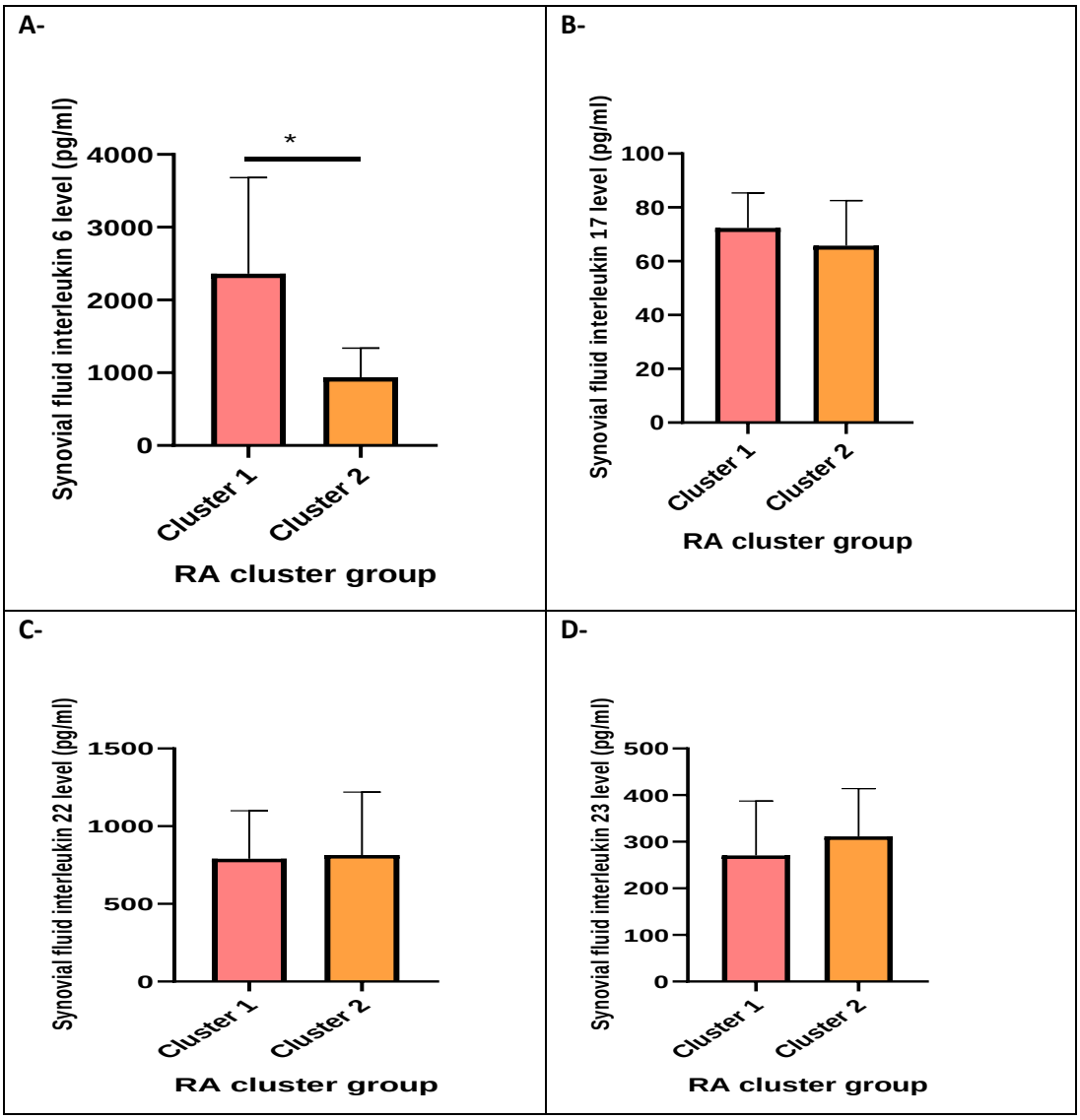


Figure 4-18 Human Magnetic Luminex Screening Assay measured IL-6, IL-17, IL-22, and IL-23 levels in the synovial fluid of the fungal RA clusters (cluster1 vs. cluster2). A, level of IL-6 was a significant increase in RA cluster1 relative to cluster2. **B-D,** IL-17, IL-22, and IL-23 levels were not significantly changed between RA cluster1 and cluster2. Values represent the mean (SD) of cytokine levels (pg/ml). The statistical significance between groups was determined by unpaired T-test, *P < 0.05.

4.4 Discussion

4.4.1 Microbiome characterisation

The microbiome is increasingly correlated with the aetiology of rheumatoid arthritis. Although most investigations have concentrated on faecal, oral, lung, and urine samples (Ebringer and Rashid, 2006; Vaahtovuo *et al.*, 2008; Zhang *et al.*, 2015; Liu *et al.*, 2016; Scher *et al.*, 2016; Wu *et al.*, 2017; Lopez-Oliva *et al.*, 2018), microenvironments inside the joints have not been systematically investigated. Recent studies have further revealed that evidence of the microbiome (generally at the nucleic acid level) is detectable in the synovial fluid of RA patients, and purports that this is the result of microorganisms (or parts thereof) moving from their usual place of the oral cavity into the synovial fluid (Karen E. Kempsell *et al.*, 2000; Moen *et al.*, 2006; Martinez-Martinez *et al.*, 2009; Ogrendik, 2009; Témoin *et al.*, 2012b; Reichert *et al.*, 2013); a process termed atopobiosis (Potgieter *et al.*, 2015). Interestingly, these studies are accompanied by evidence demonstrating the existence of these DNAs and or antibodies directed against the originating microbiome in the blood (Moen *et al.*, 2003, 2006; Lundberg *et al.*, 2008; Martinez-Martinez *et al.*, 2009; Ogrendik, 2009; Hitchon *et al.*, 2010).

Based on these results, we propose that microbiome / microbial DNA may spread in the synovial space through the blood (as demonstrated by their concurrent existence in both fluids). Extensive epidemiological and experimental evidence support that RA is caused by microbial; however, there is increasing evidence connecting the infection of the oral tissues with RA (Bartold et al., 2005; Forner *et al.*, 2006; de Pablo et al., 2008; Scher *et al.*, 2012; Brusca et al., 2014; Horliana *et al.*, 2014; Koziel et al., 2014; Cheng et al., 2017; Zhao *et*

al., 2018). In addition, there is data to show the existence of microbial DNA from distant microbial ports such as our oral cavity in the synovial space (see above). Nevertheless, there has been a limited study of the bacterial population in this compartment (Zhao *et al.*, 2018a), and no characterisation of the fungal DNA existent in this site to date, and this adds to the novelty of this research.

Here, we investigated the presence of bacterial and fungal DNA in the synovial fluid of rheumatoid arthritis (RA) patients and healthy control subjects through amplification and sequencing of the 16S rRNA V4 and ITS2 genes. Synovial fluid samples from 25 human donors were analysed in this investigation to provide an accurate description of the bacterial and fungal populations present in both RA patients and healthy subjects. It may also indicate that healthy subjects are at risk of developing RA because of the presence of a common microbial population in RA and healthy subjects.

Investigations were done at both the phylum and genus levels. At the phylum level, our results showed that *Proteobacteria* and *Firmicutes* bacterial phyla dominate synovial fluid. These phyla were also found as the most abundant in the only available comparable study, despite the slightly different proportions (Zhao *et al.*, 2018a).

At the genus level, our synovial fluid samples were dominated by the genus *Pseudomonas*, which was the common genus to be detected in the study cohort, and has previously been found in the synovial fluid of RA patients (K. E. Kempsell *et al.*, 2000; Yilmaz, Arslan and Mert, 2014; Olsen-Bergem *et al.*, 2016). We identified that while the presence of the *Raoultella* genus in RA patients is the almost significant difference (at $P = 0.051$) between the RA patients and controls, other bacterial taxa present in RA and controls were almost identical.

Genus *Raoultella* is usually considered as an opportunistic bacterium that has been isolated from the human stool, synovial fluid, saliva, sputum, and blood samples (Lam and Salit, 2014; Bonnet *et al.*, 2017; Sękowska, 2017). This is the first study to describe the genus *Raoultella* in RA synovial fluid samples; however, two common species can cause human infections: *Raoultella ornithinolytica* and *Raoultella planticola*. These are capable of causing infective arthritis (Seng *et al.*, 2016; Venus, Vaithilingam and Bogoch, 2016; Bonnet *et al.*, 2017; Levorova *et al.*, 2017). Moreover, *Raoultella* is known as histamine generating bacteria that alter histidine to histamine because of their pyridoxal phosphate-reliant on histidine decarboxylase (Kanki *et al.*, 2002). Histamine is a pro-inflammatory molecule in the pathogenesis of RA, which is existent in RA blood, articular cartilage, synovial fluid, and synovial tissue (Kim *et al.*, 2017). It has been shown that increased histamine in RA sera and synovial fluid (Kim *et al.*, 2017). Further, the genus *Raoultella* was isolated from the urine of RA patients, and the suggestion it causes urinary tract infection (Nakasone *et al.*, 2015). It has been noticed that *Raoultella* is associated with severe gastrointestinal infections; enteric translocation is regarded to be a likely approach of infection (Campos, Guimarães and Lovisolo, 2016). *Raoultella* genus may gain access to the synovial fluid from the above-mentioned infection sites, and it may also be responsible for the triggering of autoimmune reactions and causing RA.

At the phylum level, synovial fluid was dominated by members of the *Basidiomycota* and *Ascomycota* fungal phyla. These two phyla were also identified to be dominant in the blood microbiome (Panaiotov, *et al.*, 2018). Based on these findings, we suggest that fungi / fungal nucleic acid may reach the synovial location via the blood (as evidenced by their concurrent existence in both fluids).

At the genus level, synovial fluid samples were predominated by the genus *Malassezia*, which accounted for 40.9% and 45.3% of the total number of fungal sequences identified in

the RA and control donors, respectively. Differences in the structure of the fungal communities present in synovial fluid were consistently identified between RA patients and healthy controls. We have identified that the order *Hypocreales* was significantly more abundant in the synovial fluid of RA patients and absent from the synovial fluid of healthy control. The members of the order *Hypocreales* have been previously found in the oral cavity of healthy humans (Peters *et al.*, 2017a) and identified to be ubiquitous in human blood (Beatty *et al.*, 2014; Panaiotov *et al.*, 2018). *Hypocreales* may reach the synovial fluid from these sites. However, no reports directly associate *Hypocreales* with RA.

Furthermore, a member of the genus *Aspergillus* was observed almost exclusively in RA synovial fluid samples only (P = 0.14, including all data, P = 0.057 excluding samples where <1% of reads mapped to this taxa). *Aspergillus* is present in the healthy human gut (Hallen-Adams and Suhr, 2016) and oral cavity (Ghannoum *et al.*, 2010; Peters *et al.*, 2017b). In several studies have been found that *Aspergillus* is a prevalent opportunistic pathogen of the lungs, particularly affecting immunocompromised individuals, and it causes a range of pulmonary conditions such as allergic bronchopulmonary disease and invasive pulmonary aspergillosis (Singh *et al.*, 2003). It has been identified that the treatment with Adalimumab (an anti-TNF drug, which is utilized to treat some inflammatory diseases including rheumatoid arthritis) has been linked with various fungal infections, including Aspergillosis (Tsiodras *et al.*, 2008; Manz et al., 2009; Salmon-Ceron *et al.*, 2011; Kawaski *et al.*, 2016; Ferrer *et al.*, 2018).

Adalimumab is a recombinant human immunoglobulin monoclonal antibody that attaches to the soluble and transmembrane form of TNF-α with high affinity, thus blocking the interaction of TNF-α with its receptors (Lim *et al.*, 2018). TNF-α is not merely a mediator of inflammation yet also an integral part of the normal defence response against infection. It is included in the recruitment of inflammatory cells to the site of infection, induction of other

inflammatory cytokines, apoptosis, and cell activation (Kim *et al.*, 2011). It has indicated that patients treated with TNF-α inhibitor such as Adalimumab have a decreased interferon-γ production and reduced expression of Toll-like receptor-4, which is important for the recognition of microorganisms via dendritic cells and phagocytes (Giles and Bathon, 2004). Therefore, the use of Adalimumab is predicted to enhance susceptibility to fungal infections (Bakleh *et al.*, 2005; Tsiodras *et al.*, 2008; Kim *et al.*, 2011; Kobak *et al.*, 2014; Lim *et al.*, 2018).

Moreover, Serum *Aspergillus galactomannan* antigen levels are frequently increased in a nonspecific manner in RA patients (Horie *et al.*, 2016).

Relative abundances of unclassified organism belonging to the order *Malasseziales* and genus *Cladosporium* were significantly reduced in RA patients relative to healthy controls. Unclassified organism belonging to the order *Malasseziales* has been detected in the healthy human blood (Panaiotov, Filevski, Equestre, Nikolova, Kalfin and Panaiotov, 2018), gut (Hallen-Adams and Suhr, 2016), oral cavity, nares, and respiratory tract (Witherden *et al.*, 2017). Furthermore, *Malasseziales* was identified to differ in abundance in the case of inflammatory bowel condition (Sokol *et al.*, 2017; Witherden *et al.*, 2017), proposing it may be responsive to an inflammatory environment. *Cladosporium* has been detected in the healthy human gut (Hallen-Adams and Suhr, 2016), and oral cavity (Peters *et al.*, 2017b), and is encountered commonly in human clinical samples (Sandoval-Denis *et al.*, 2015); thus, proof of *Cladosporium* DNA in the healthy patient is possibly not unexpected. That said, it's absence from the synovial fluid of all RA patients may propose the changed fungal population in some distant microbial niche, which presents as a reduction in translocated *Cladosporium* DNA. These results all warrant further investigation.

Our results revealed that gender and age could be co-influence factors on the microbiome population in RA synovial fluid. The member of *Enterococcus*, *Stenotrophomonas* genera were found almost exclusively in RA synovial fluid samples from men only when compared those to women. Further, the abundance of *Raoultella* and *Hypocreales* as marginally significant; they are identified as an increase in males RA synovial fluid samples compared to females. Moreover, an abundance of *Raoultella* and *Hypocreales* were significantly increased in RA patients with age ≤ 65 compared to RA patients with >65. Most of these taxa have not been associated with gender and age before in human studies, with the notable exception of *Raoultella*, which was also increased in men in a recent study (Insenser *et al.*, 2018).

Gender and age are the significant variables influencing microbiome composition; several investigations have observed gender and age differences in microbiome diversity and composition (Yatsunenko *et al.*, 2012; Jašarević, Morrison, and Bale, 2016; Martin *et al.*, 2016; Johnson, 2020).

Gender differences in the evolution and presentation of different diseases, such as RA, have been known. However, the associated mechanism is unclear (Gomez *et al.*, 2012; Danska, 2014; Bodkhe, Balakrishnan, and Taneja, 2019). Gender differences in the intestinal microbiome may play a role in the gender differences in illnesses (Danska, 2014).

The gender differences in the innate and adaptive immune systems are well understood (Klein and Flanagan, 2016). Receptors for sex hormones are expressed on most immune cells. Thus sex hormones may play a role in establishing the gender difference in the immune response (Elderman, de Vos and Faas, 2018). Because the gut microbiome interacts with the host immune system, it can be expected that the gender differences in the intestinal microbiome have some role in the gender differences in immunity (Fransen *et*

al., 2017). Intestinal inflammation might also have gender differences about the gut microbiome. In a mouse model of colitis induced with 2, 4, 6-trinitrobenzene sulfonic acid, the males showed more severe colonic inflammation (Kozik *et al.*, 2017).

Probiotics also caused different inflammatory responses from female and male mice (Lee et al., 2017). In female Wistar mice presented to water avoidance stress, the administration of Lactobacillus farciminis significantly lowered the colonic mucosal mast cell count and decreased the levels of inflammatory cytokines only in the female mice (Lee et al., 2017). Besides, sex differences in response to probiotic Lactobacillus animalis NP-51 administration were recorded for cytokine responses, intestinal metabolic profiles, and intestinal microbiome in Mycobacterium-treated mice (Karunasena *et al.*, 2014).

The FMT animal model of another study showed that female recipients lost significantly more weight after taking the male microbiome when compared to those with the weight after taking the female microbiota, proposing that the male microbiome caused more gut inflammation (Fransen *et al.*, 2017).

Age also alters the intestinal microbes, and then this may cause alteration anther human microbiota, such as the synovial fluid microbiome. This may occur because older people eat a less complicated diet and are expected to take multiple medications that may change the microbes in their intestine (Ghosh *et al.*, 2020). Because of this, age may influence variations in intestinal microbes associated with illnesses(Ghosh *et al.*, 2020). This highlights the need for studies that tease apart the importance of ageing-related and disease-related changes in the gastrointestinal microbiome(Ghosh *et al.*, 2020).

Ghosh et al. (Ghosh *et al.*, 2020) indicated that intestinal microbe variations associated with diseases might vary with a person's age. The analysis compared the intestinal microbiomes of more than 2,500 people aged 20 to 89(Ghosh *et al.*, 2020). This included

people with type 2 diabetes, inflammatory bowel disease, liver cirrhosis, colorectal cancer, and intestinal polyps(Ghosh *et al.*, 2020). The study found that younger people gradually gain disease-associated intestinal microbes. In comparison, older people tend to lose the gut microbes, usually observe in a healthy intestine(Ghosh *et al.*, 2020). Ghosh et al. (Ghosh *et al.*, 2020) also found a set of gut microbes that were gained in several diseases and across age-groups. This set of microbiota was also associated with frailty in older individuals. The characteristics of the microbiota in this set are all distinguished to have detrimental impacts on human health (Ghosh *et al.*, 2020).

Several environmental factors, such as antibiotics, diet, smoking, stress, and geographical location, can influence both the microbiome diversity/composition and the arthritis onset/outcome(Diamanti *et al.*, 2016; Bodkhe, Balakrishnan and Taneja, 2019b). The utilisation of antibiotics is a two-edged weapon: it kills both beneficial and pathological microbes indiscriminately, allowing the loss of intestinal microbiota or the so-called dysbiosis and increase of pathogenic microbiota (Hasan and Yang, 2019). Investigations on experimental mice have shown the administration of the antibiotic affected secondary bile acid and serotonin metabolism in the colon and producing in delayed intestinal motility through providing microbiota reduction (Ge *et al.*, 2017; Hasan and Yang, 2019). Antibiotics disrupt the competitive exclusion machinery, a fundamental property by which microbiotas eliminate pathological microorganisms (Hasan and Yang, 2019). This disruption increases the growth of other pathogens, for instance, Clostridium difficile (Ramnani *et al.*, 2012). Studies have recorded that clarithromycin and metronidazole (Jakobsson *et al.*, 2010), clindamycin (Jernberg *et al.*, 2007), and ciprofloxacin (Dethlefsen and Relman, 2011) influence the microbiota structure for a long time.

The relevance of diet is suggested by the differences in the gut microbiota between geographically and lifestyle distant populations (Diamanti *et al.*, 2016). In general,

individuals assuming a diet rich in animal proteins, simple sugars, and fats have a decreased diversity of intestinal microbiota with a predominance of *Bacteroidetes taxa* (Diamanti *et al.*, 2016). Dietary intervention, fish oil, reduction in animal protein intake, among others, can modify microbiome diversity in the intestine, sometimes leading to a mild decrease of joint inflammation *taxa* (Diamanti *et al.*, 2016).

Smoking can impact on the gut, and periodontal microbial populations may represent risk factors for the development of RA (Huang and Shi, 2019). Few are the investigations that have investigated the impact of smoking on the composition of the gut microbiome. However, Biedermann et al. (Biedermann *et al.*, 2013) have recently shown significant alterations after smoking cessation with an increase in Actinobacteria and Firmicutes, accompanied by a decrease of *Bacteroidetes*. In PD, the effect of smoke on the microbiome is controversial. However, it has been identified that T. forsythia, P. gingivalis, Aggregatibacter actinomycetemcomitans, Parvimonas, and Treponema have a higher predominance on the oral of smokers relative to non-smokers subjects (Zambon *et al.*, 1996; Shchipkova, Nagaraja and Kumar, 2010; Jiang *et al.*, 2020)

It is understood that stress can alter the immune function and, consequently, autoimmune disorders through different serological and cellular pathways; a clinical role for stress in the onset and flares of patients with chronic arthritis has been recorded (Stojanovich and Marisavljevich, 2008; Diamanti *et al.*, 2016). However, it requires to be better explained. The axis between the brain and immune system also involves the microbiota as established by investigations using several animal models and by the recent description of intestinal microbiota alterations in patients with depression and irritable bowel syndrome (Diamanti *et al.*, 2016; Wang and Wang, 2016; Martin *et al.*, 2018; Cheung *et al.*, 2019).

The microbiome composition may differ by geographical location for several causes. Microbial and environmental pressures can change both the repertoire of bacterial species inhabiting the region and their abundance (Arrieta *et al.*, 2014; Gupta, Paul and Dutta, 2017; Hasan and Yang, 2019). Different ethnographic populations have distinct regional diets, genetic backgrounds, and cultural practices. Of course, resource-replete areas also have entrance to better hygiene and healthcare than developing nations (Arrieta *et al.*, 2014). Therefore, when investigations are designed to evaluate geographical differences in microbiome populations, any trends are attributable to a large body of varieties other than geographical separation (Arrieta *et al.*, 2014). Still, comparisons between several developed and developing areas have provided some insight into which geography-associated variables are the most reliable drivers of microbial diversity (Arrieta *et al.*, 2014).

A comparison between continentally distinct populations led to the emergence of different types of population structures, driven by the composition of the *Bacteroidetes* phylum (Arumugam *et al.*, 2011; Arrieta *et al.*, 2014). The predominant genera identified French, Japanese, Danish, Spanish, Italian, and American individuals in the phylum being either *Bacteroides* or *Prevotella* or with a less pronounced Ruminococcus signature (De Filippo *et al.*, 2010; Arrieta *et al.*, 2014).

A major limitation of the study we cannot compare synovial fluid profiles with blood/sera from chapter 3 because they are not the same patients to evaluate the effects of microbial dysbiosis at the blood on the synovial fluid microbiome.

4.4.2 **Alterations synovial fluid cytokine levels in RA**

Numerous cytokines have been involved in RA pathology. Many have the possibility as markers in RA, particularly as their clinical utility is previously established in other illnesses and could be easily transferable to rheumatology (Burska, Boissinot, and Ponchel, 2014). Higher concentrations of IL6, IL-17-A, IL-22, and IL23 have been observed in the synovial fluid of RA patients compared with healthy control subjects. The data publicised by a number of authors in the literature agreed with our findings. Over-production of IL-6 was previously identified in synovial fluid and RA blood, and IL-6 levels were correlated with illness activity (Srirangan and D. E. H. Choy, 2010; Boyapati *et al.*, 2019; Rajaei *et al.*, 2019). IL-6 has an essential role in the pathogenesis of RA, and current evidence indicates that the blockade of IL-6 is an effective treatment for patients with RA (McInnes, Buckley and Isaacs, 2016).

IL-6 has a vital immunoregulatory role in adaptive immunity, acting at a checkpoint in regulatory T (TREG) cell pathways and the differentiation of naive T cells towards pro-inflammatory TH17 cells (McInnes, Buckley and Isaacs, 2016). Matsumoto et al. (Matsumoto, Tsurumoto and Shindo, 2006) has identified that a positive correlation between the concentration of IL-6 in synovial fluids and the infiltration of the inflammation cells in synovial tissues, proposing IL-6 in the synovial fluid appears to be an excellent marker to reveal the infiltration of the inflammation cells in the synovial membrane. Sack et al. (Sack *et al.*, 1993) showed that the synovial fluid IL-6 levels were associated with histological characteristics of chronic synovitis in RA. These results support the notion that IL-6 is an important cytokine for stimulating synovial inflammation.

Previous results found higher synovial fluid IL-17A levels in RA patients relative to healthy controls. Synovial fluid levels of IL-17A could be utilized as a potential systemic biomarker for RA patients. Previous results found higher synovial fluid IL-17A levels in RA patients relative to healthy controls (Roşu *et al.*, 2012; Elhewala *et al.*, 2015). The archetypal proinflammatory T-cell cytokine, IL-17A, has multiple overlapping functions with both TNF and IL-6. IL-17A in synovial fluid of RA patients stimulates the release of proinflammatory cytokines (involving TNF, IL-1, and IL-6), chemokines (CXC-chemokine 8 and CC-chemokines 2 and 3) and facilitating osteoclast activation and angiogenesis (the formation of new blood vessels) (McInnes, Buckley and Isaacs, 2016). Therefore, Synovial fluid levels of IL-17A could be utilised as a potential systemic biomarker for RA patients.

The IL-22 level in synovial fluid was higher in RA patients relative to healthy controls (da Rocha *et al.*, 2012b; Xie, Wang, and Li, 2012). It has been found that levels of interleukin 22 were elevated in RA patients relative to controls. Further, IL-22 levels associated with disease activity, rheumatoid factor positivity, was associated with higher levels of IL-22 in RA patients, and the existence of bone erosions was correlated with high IL-22 levels (da Rocha *et al.*, 2012b). These results propose that IL-22 may be a biomarker for estimation of activity in RA, and IL-22 appears to be a possible therapeutic target for RA.L-23 levels in synovial fluid are high in RA patients (Yago *et al.*, 2017), and IL-23 may be a beneficial biomarker for the diagnosis of RA. It has been known that systemic levels of IL-23 are strongly related to illness activity in RA (Melis *et al.*, 2010). IL-23 is supposed to endorse the swelling and destruction of joints in patients with RA through two processes: (1) inflammation and (2) bone destruction. The mechanism of these two effects is caused by IL-23-induced stimulation of IL-17 (Yago *et al.*, 2017).

We identified that IL-6 levels were higher among a sub-cluster of the RA population defined according to the fungal and bacterial microbiome. IL-6 has been found to play a pivotal role

in the RA pathogenesis (Narazaki, Tanaka, and Kishimoto, 2017a). It is involved in the pathogenesis of the local joint illness and systemic manifestations (Srirangan and E. H. Choy, 2010; Yoshida and Tanaka, 2014b). The level of synovial fluid IL-6 has been seen associated with illness severity and activity (Matsumoto, Tsurumoto and Shindo, 2006; Rajaei *et al.*, 2019). Fungal pathogen is known to activate IL-6 expression while the precise mechanism of initiation and persistent expression of IL-6 in RA patients is still being studied (Narazaki, Tanaka and Kishimoto, 2017a). In fungal pathogen-infected lesions, IL-6 is generated via the activation of toll-like receptors (TLRs) on macrophages and dendritic cells with the TLR-recognising microbial components, which are known as pathogen-associated molecular patterns (PAMPs). Various fungal populations are distinguished via cell-surface TLRs. This cell-surface induce IL-6 and other inflammatory cytokines (Kawai and Akira, 2010). An in vitro study identified that in response to auto-antigen more IL-17A, IL-6, and IL-23 were generated via naïve T cells cocultured with RA-microbiota activated dendritic cells than by T cells cocultured with healthy control-microbiota activated dendritic cells, proposing that dysbiosis of the microbiome may be involved in the pathogenesis in RA (Maeda *et al.*, 2016). Furthermore, Th17 cells are fundamental in the immune response against fungal pathogens, and IL-6 is implicated in the differentiation of lymphocytes to Th17 cells (Srirangan and E. H. Choy, 2010). This suggests that the variation in the fungal microbiome within RA patients might be associated with illness severity by an IL-6 – Th17 mediated pathway.

4.5 Conclusion

This study confirmed the existence of fungal and bacterial DNA in synovial fluid and determines that this is changed by disease condition (RA), as are other classical microbiome niches. It anticipated that the microbiome originates from one of the classical microbiome niches (the gut, mouth, urogenital tract, skin) and enters the blood, which acts as a conductor between their usual place of habitation and the synovial place. Gender and age could be co-influence factors on the microbiome population in RA synovial fluid.

Chapter 5

5 Molecular characterisation of the stool, synovial fluid, urine, blood, and serum of collagen-induced arthritis mouse model and healthy Control subjects

5.1 Introduction

Mouse and human are quite similar in physiology and anatomical structures, and this is one of the reasons why mouse models have been widely utilised in biomedical studies. Mainly, the intestinal tracts in both species are composed of anatomically similar organs (Hugenholtz and de Vos, 2018). Further, their low maintenance cost (relative to other mammalian experimental models), high reproductive mice, and short life cycle are valuable advantages of the mouse model (Nguyen *et al.*, 2015). Steps on this journey require the characterisation of the current available RA models, the CIA being on a ubiquitous option. Collagen-induced arthritis (CIA) can be induced in DBA1 mice via high-quality collagen. CIA shares several similarities pathological and immunological features with human RA and has been extensively studied to explore pathogenesis as well as test candidate treatments (Brand *et al.*, 2007). Clinical signs of CIA typically develop after the initial inoculation and appear as polyarthritis. This is the most prominent in the limbs and characterised by synovial inflammatory infiltration, bone and cartilage and erosion, and synovial hyperplasia similar to human RA (Asquith *et al.*, 2009; Choudhary *et al.*, 2018). This initiates an immune reaction directed towards collagen, including activation of T cells and B and antibody production. Antibodies distinguish collagen in the joints and stimulate the local autoimmune response, in which T cells, monocytes, granulocytes are attracted to the joints. This further fuels the inflammatory process, leading to the production of cytokines and inflammatory mediators (Choudhary *et al.*, 2018).

Disease severity is expected to peak at approximately day 35, after which DBA/1 mice enter remission, marked by increased concentrations levels of serum IL-10 and a subsequent lowering in pro-inflammatory Th1 cytokine (Asquith *et al.*, 2009).

DBA/1j male mice and bovine CII was used to induce the collagen-induced arthritis model (Joosten and van den Berg, 2006). Male mice exhibit higher susceptibility relative to female mice (Joosten and van den Berg, 2006). Bulk quantities of bovine CII can be taken from articular cartilage slices obtained from a knee joint of 1–2-yr old cows (Asquith *et al.*, 2009). The onset of arthritis begins around days 25–28, often first affecting some digits of forepaws and hind, then spreading to multiple places in the paw, including the joint parts. Affected joints undergo devastating consequences (Asquith *et al.*, 2009; Choudhary *et al.*, 2018). The commonly hypocellular synovium becomes infiltrated with immune cells (macrophages, neutrophils, T cells, and B cells) (Choudhary *et al.*, 2018). This leads to the formation of pannus, a hyperplastic membrane of synoviocytes that shows a tissue-invasive character, targeting bone and cartilage (Choudhary *et al.*, 2018). Generation of matrix-degrading enzymes, which are generated by activated synoviocytes and chondrocytes, and infiltrating mononuclear cells such as neutrophils, destroy cartilage (Choudhary *et al.*, 2018).

In addition, nitric oxide generated by macrophages and synoviocytes induces the destruction of chondrocytes. Besides, a variety of cytokines and growth factors, including IL-1β, IL-17, TNF-α, and macrophage colony-stimulating factor generated by synoviocytes, T cells, B cells, and monocytes induce the expression of RANKL and bone erosion activity (Choudhary *et al.*, 2018).

Moreover, the production of osteoclasts are detected in the area of focal bone erosion in vivo in CIA mice, produces bone erosion. The synovial fluid, which usually contains few

cells, becomes highly infiltrated, predominantly with neutrophils (Choudhary *et al.*, 2018). Together, these modifications result in decreased functioning of the joints characterised by pain and stiffness.

Multiple cytokines have been involved in the pathogenesis of the CIA, such as TNF-alpha, IL-1beta, and IL-6 (Marinova-Mutafchieva *et al.*, 1997). TNF-alpha-expressing cells were identified on day 1 of arthritic disease, whereas IL-1b-expressing cells were not shown until day 3 of disease, proposing that the expression of TNF alpha leads that of IL-1 (Marinova-Mutafchieva *et al.*, 1997). There was a progressive expansion in the number of TNF-alpha, IL-1beta, and IL-6-positive cells from day 1 to day 10 of disease, through which time IFN-gamma production via CD4+T cells from draining lymph nodes decreased sharply (Marinova-Mutafchieva *et al.*, 1997).

IL-1beta and IL-6 levels, but not TNF-alpha in paw tissues significantly induced between day 14 and day 28 following collagen immunisation, when the disease was at a developed stage (Magari *et al.*, 2004). It was noted that treatment with a set of neutralising antibodies against both IL-1 alpha and IL-1 beta was still highly effective in established arthritis, decreasing both inflammation and also the progression of cartilage damage (Dinarello, Simon and Van Der Meer, 2012).

Studies characterising of the CIA microbiome following the induction of RA are rare. Liu and co-workers (Liu *et al.*, 2016) identified that the gut microbiome of CIA-susceptible and CIA-resistant mice were modified in a phenotype-specific manner resulting in illness induction. In addition, when the microbiota of CIA-susceptible mice colonised germ-free mice, it brought about a considerable increment in RA incidence and severity (Liu *et al.*, 2016). Taken together, these facts, it is proposed that collagen therapy is not enough for establishing arthritis and highlights the role of contributing to the intestinal microbiome. In

this chapter, we attempted to characterise the gut, synovial fluid, urine, blood, and serum of CIA in comparison to controls mice subjects to confirm the gut dysbiosis, and then to endeavour in the same animals for signatures of these taxa in the blood, synovial fluid, and urine. This study was performed to link dysbiosis in multiple microbiome niches in the collagen-induced arthritis model following the induction of experimental RA.

5.2 **Methods**

Twenty mice in total, with an aged eight-week-old male, were used in this study. Between these, ten were from DBA1 mice with CIA (this model by immunisation with an emulsion of complete Freund's adjuvant and type II collagen (CII) in the tail on Day 1 and Day 21 (Williams, 2007). For control animals (n=10), Phosphate-buffered saline (PBS) was injected instead of bovine type II collagen.

Eighteen days posted the second injection when inflammation was satisfactory, the faeces, urine, synovial fluid, blood, and serum were collected from mice.

Methods for DNA extraction step, 16S rRNA amplification, Gel electrophoresis, DNA purification by Qiagen Purification Kit, Addition of Illumina XT tags, DNA purification by AMPure XP magnetic beads, DNA sequencing utilising an Illumina MiSeq, and subsequent bioinformatic analysis were found in the **Methods chapter**.

5.3 Results

5.3.1 Bodyweight, arthritic Index, and hind paw thickness

Mean animal bodyweight, arthritic index, and hind paw thickness were checked three times per week.

1-Bodyweight

The body weights of all mice in the study were measured three times per week. No animal lost >10% of its body weight throughout the study period **(Figure 5-1).** The statistical analysis by T-test showed that there was a significantly reduced weight of CIA mice **(P<0.01)** in comparison to controls. Recently, Zhang and his co-works mentioned that the weight of CIA mice decreased significantly relative to healthy controls (Zhihui Zhang *et al.*, 2019). Further, it has been observed that severe weight loss during the early RA period was linked with an increased subsequent mortality risk for RA patients (Sparks *et al.*, 2018).

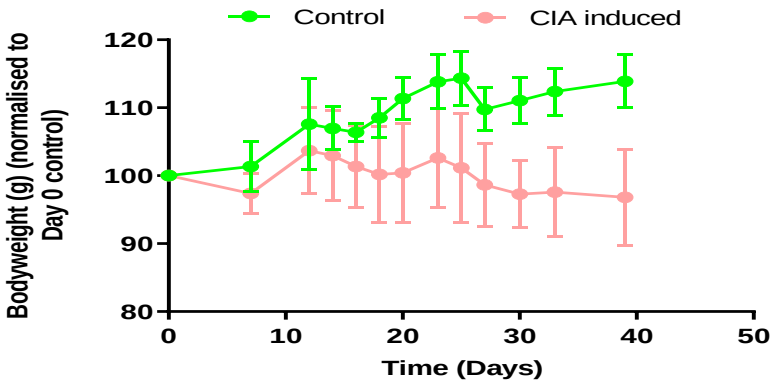


Figure 5-1 Mean (SD) bodyweight of male DBA/1 mice with induced arthritis compared to control mice. Bodyweight (g) was monitored three times per week. n=10 for all groups.

2-Arthritic Index

The arthritic Index of all mice in the study was measured three times per week.

The statistical analysis by T-test revealed that the arthritis score was high in the CIA cohort **(P<0.01)** relative to controls. CIA induced animals were ranging within an arthritic index of 3-4 (except for animal 1), indicative of severe inflammation and established arthritis **(Figure 5-2).**

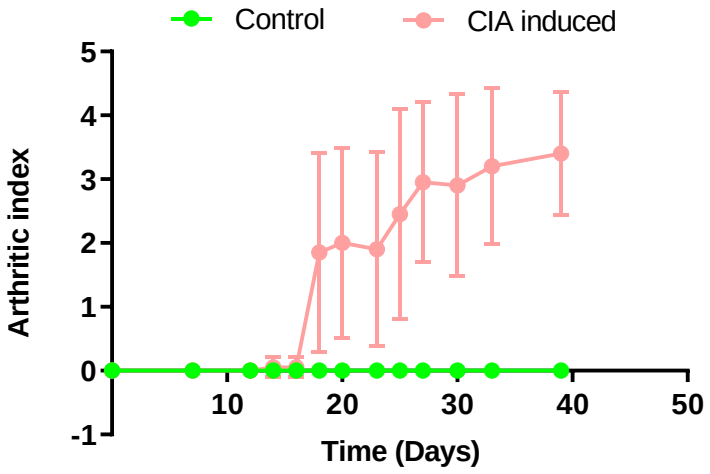


Figure 5-2 Mean (SD) arthritic index of male DBA/1 mice induced arthritis compared to control mice. Paw thickness was measured three times per week. n=10 for all groups.

3-Hind Paw Thickness

As an additional measurement of disease severity, the mean thickness of hind paws was measured for each animal three times per week. The statistical analysis by T-test found that Paw Thickness of male DBA/1 mice induced arthritis was increased significantly **(P<0.01)** compared to healthy controls. These measurements followed a similar pattern to that observed with the Arthritic Index **(Figure 5-3).**

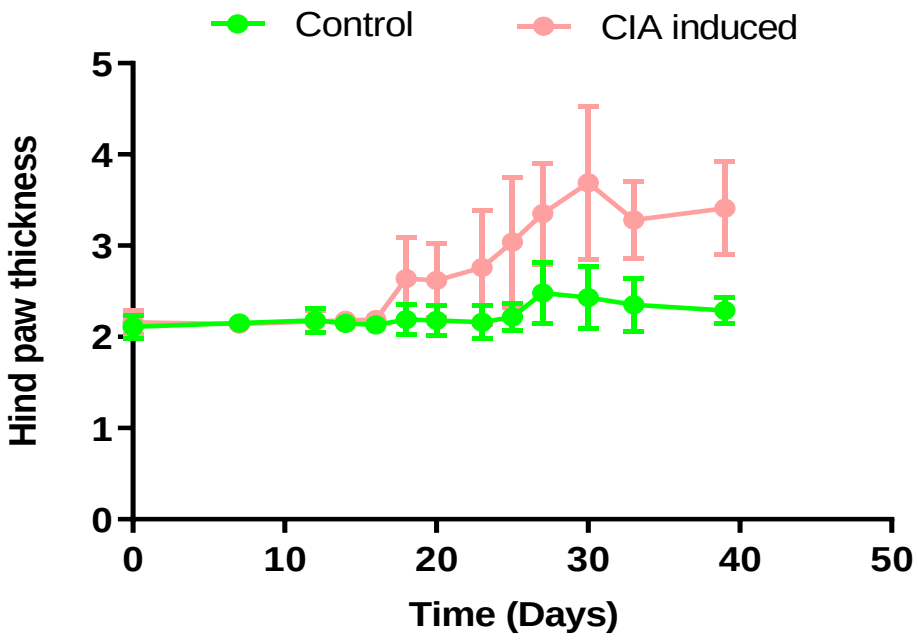


Figure 5-3 Mean (SD) Paw Thickness (mm) of male DBA/1 mice induced arthritis compared to control mice. Paw thickness was measured three times per week. n=10 for all groups.

5.3.2 **Characterisation of the intestinal bacteria in mice**

Eighteen days posted the second injection (terminal stage of an experiment), the faeces, was harvested from mice.

The characterisation of bacterial DNA in the mice stool was evaluated via PCR amplification and sequencing of the bacterial 16S rRNA gene, variable region 4. An average of 99,244.100 reads was generated for each of the samples; 103,120 reads in the CIA samples, and 95,368.2 reads in the Control samples. Although the CIA samples generated more reads on average, this difference was not statistically significant (P >= 0.05), followed by bioinformatic analysis using QIIME **(See 2.3.4)**. Our first approach used principal coordinates analysis (PCoA) to reduce the complexity of the data obtained and to immediately visualise any obvious clustering between the two experimental groups **(Figure 5-4).**

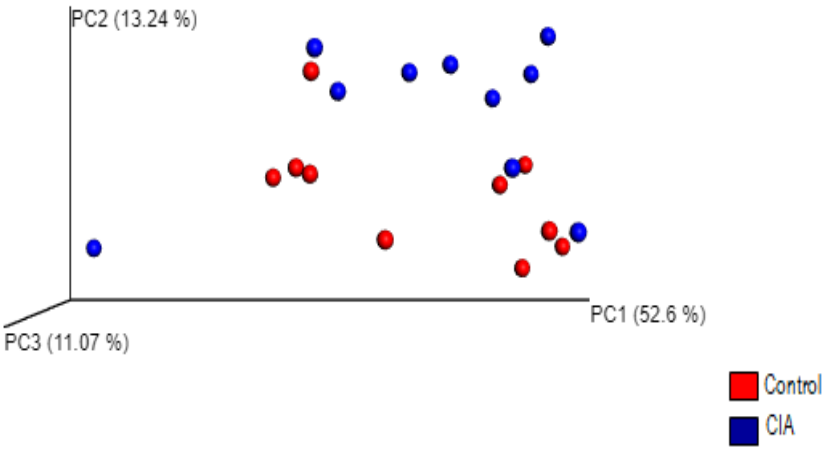


Figure 5-4 PCoA plot informed by weighted unifrac distance matrix for CIA mice (blue) and untreated controls (red). Distance matrix was informed by amplification and sequencing of the 16S rRNA variable region 4, followed by a taxonomic assignment. Proportions of variation explained by the principal coordinates are designated on the axes. PCoA found that the maximal variation was 52.6% (PC1), 13.24% (PC2) and, 11.07% (PC3). The microbiota of samples that appear in close proximity to each other is more similar in composition.

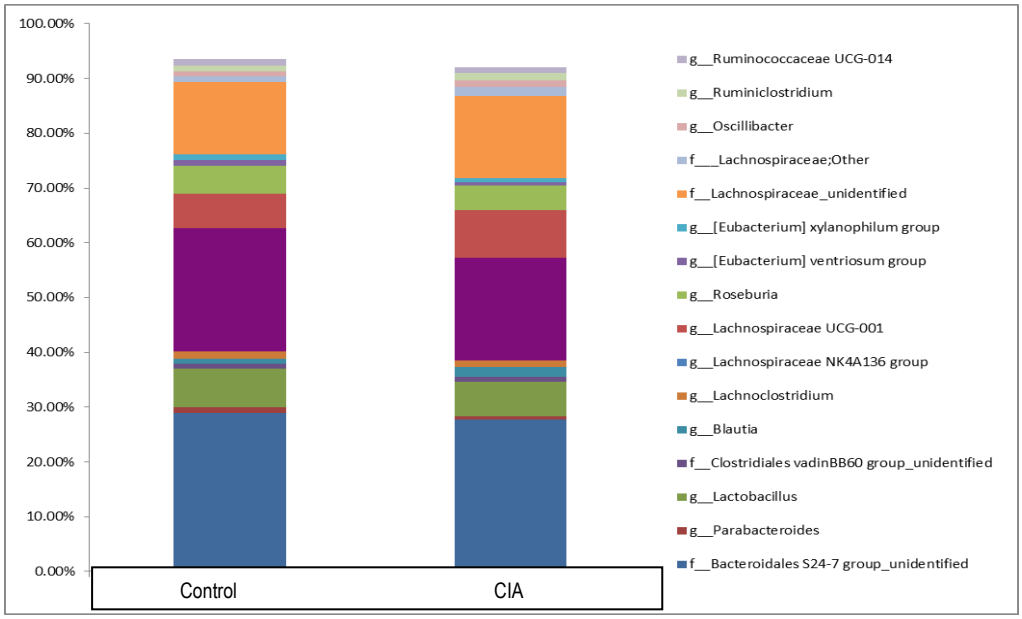
5.3.3 **Microbiome Community Composition of faecal samples**

Our various experimental negative controls (the negative Controls of DNA extraction and purification step by PCR Purification Kits/ controls of PCR experiments) constantly failed to generate a visible band after PCR and agarose gel electrophoresis. Besides, DNA quantification utilising the Qubit 3.0 high-sensitivity DNA kit (Invitrogen), confirmed this absence of DNA from the negative controls. The Qubit 3.0 high-sensitivity DNA kit is designed to be accurate for initial sample concentrations from 10 pg/µl. However, 0 values were obtained.

Our characterisation of the bacterial 16S rRNA present in the fecal samples found that the majority of bacterial 16S RNA belonged to the *Firmicutes* phylum (total relative abundance = 69.0%; Control mean = 68.4%; CIA mean =69.6%), and the *Bacteroidetes* phylum (total relative abundance = 29.4%, Control mean= 29.9%, CIA mean = 28.8%).

At the genus level **(Figure 5-5),** fecal samples were predominated by an unidentified member of the *Bacteroidales S24-7 group* (CIA= 27.7%, Control = 28.9%), followed by genus *Lachnospiraceae NK4A136 group* (CIA = 18.8%, Control = 22.4%), and an unidentified member of the Lachnospiraceae (there were other Lachnospiraceae group taxa that were found, and identified the read numbers do not relate to that genus per sequene, but specific taxa within it) (CIA = 15%, Control = 13.1%). To a lesser extent, the faecal samples contained genera *Lachnospiraceae UCG-001* (CIA = 8.7%, Control = 6.3%), *Lactobacillus* (CIA = 6.3%, Control = 7%), *Roseburia* (RA = 4.5%, Control = 5.2%), *Blautia* (CIA = 1.8%, Control = 0.8%), *Lachnoclostridium* (CIA = 1.2%, Control = 1.4%), R*uminiclostridium* (CIA = 1.4%, Control = 1%), *Ruminococcaceae UCG-014* (CIA = 1%, Control = 1.2%), and *Oscillibacter* (CIA = 1.1%, Control = 0.9%).

A-



B-

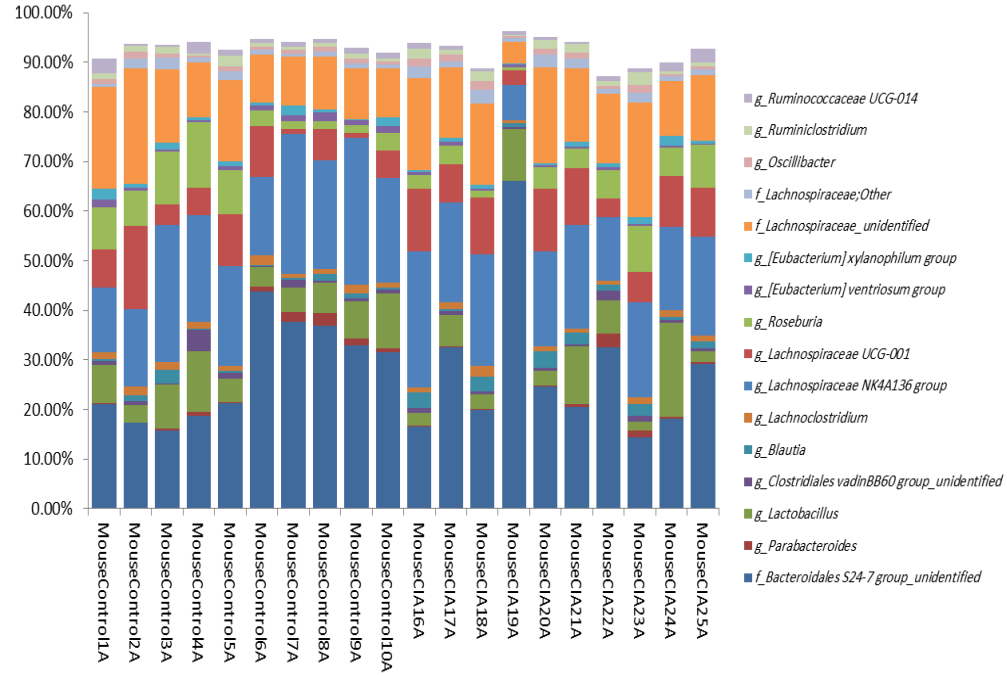


Figure 5-5 Relative abundance of genera detected within faecal samples. Data are the relative abundance of the major bacterial taxa, characterized as having a mean abundance of >1% of the total bacteria content in any one experimental group, detected in the faeces of CIA mice (CIA, n = 10), and Control (Control, n = 10) samples as determined using amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean abundance expressed as a percentage of the total bacterial sequence count. **A-** Taxa data grouped by condition and **B-** Taxa individual sample data.

The CIA and healthy controls data for all taxa were then analysed. We found that the CIA mice were associated with significantly increased levels of genera *Acetatifactor, Alistipes, Blautia, Coprococcus 1,* and *Marvinbryantia* and significantly decreased levels of genera *[Eubacterium] ventriosum group, Ruminococcaceae UCG-005, Ruminococcus 1*, and [*Eubacterium] coprostanoligenes* group when compared those with our untreated controls **(Figure 5-6 and Table 5-1).**

Table 5-1 Taxa significantly are altered by disease status. Data are median (SD). **P values were determined by applying a two-tailed, Mann Whitney test using GraphPad Prism V8. P ≤ 0.05 was considered statistically significant.**

Taxa	Control abundance Median (SD)	CIA abundance Median (SD)	P value
g__Acetatifactor	0.2 (0.19)	0.7 (0.6)	**0.0002**
g__Alistipes	0 (0)	0.2 (0.8)	**0.01**
g__Blautia	0.45 (0.8)	1.8 (1.1)	**0.019**
g__Coprococcus 1	0.1 (0.03)	0.2 (0.1)	**0.0007**
g__Marvinbryantia	0 (0.05)	0.1 (0.16)	**0.005**
g__[Eubacterium] ventriosum group	1 (0.4)	0.45 (0.15)	**0.0007**
g__Ruminococcaceae UCG-005	0.2 (0.06)	0.1 (0.04)	**0.035**
g__Ruminococcus 1	0.2 (0.07)	0.05 (0.05)	**0.0006**
g__[Eubacterium] coprostanoligenes group	0.05 (0.09)	0 (0)	**0.032**

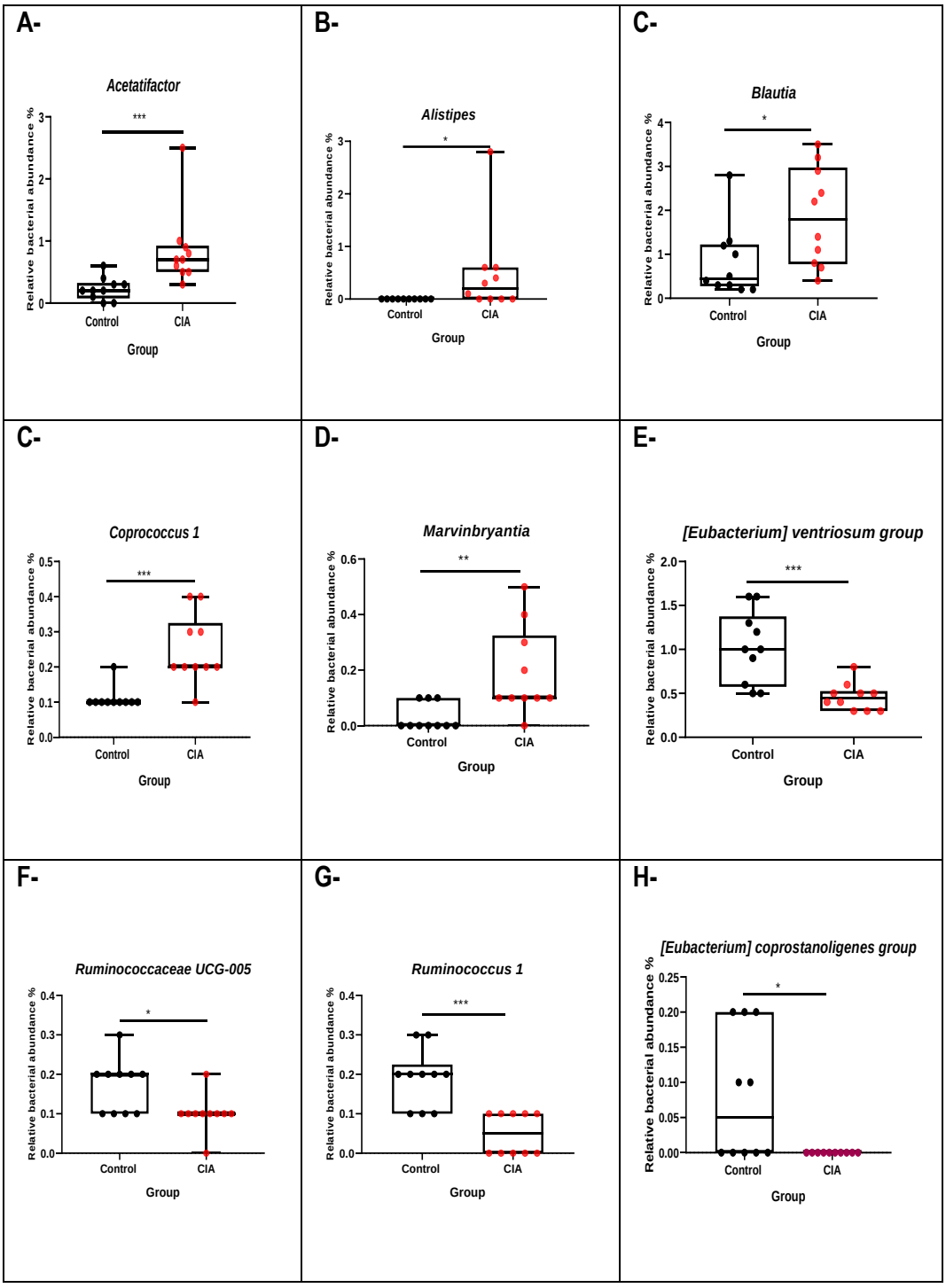


Figure 5- 6 Relative abundance of significantly changed taxa found in the mice faeces with collagen-induced arthritis and untreated controls. Data determined by the amplification and sequencing of the 16S rRNA gene variable region (V4). Data are a median abundance expressed as a percentage of the total bacterial sequence count.

5.3.3.1 The correlation between the gut microbiome genera associated with the CIA and loss of body weight

Here, we investigated whether genera significantly associated CIA are correlated with changed body weight in the CIA at terminal microbiome measurements (Eighteen days posted the second injection) to determine if taxa signatures are related to body weight loss. The taxa include *Acetatifactor, Blautia, Coprococcus 1, Marvinbryantia, Alistipes, Eubacterium ventriosum group, Eubacterium coprostanoligens, Ruminococcus 1, and Ruminococcaceae UCG-005.*

Spearman's rank correlation revealed that no such association was found to exist among *Acetatifactor, Blautia, Coprococcus 1, Marvinbryantia, Alistipes, Eubacterium ventriosum group, Eubacterium coprostanoligens, Ruminococcus 1, and Ruminococcaceae UCG-005* with changed body weight in the CIA **(Table 5-2).**

These data propose that there was no association between the gut dysbiosis in the CIA and body weight.

Table 5-2: Spearman's rank correlation coefficient determines the correlation of intestinal genera significantly associated with CIA status with the change of body weight

Bacterial taxa vs. body weight	Spearman's rank correlation	P value
g__Acetatifactor vs. **body weight**	-0.14	ns
g__Alistipes vs. **body weight**	0.3	ns
g__Blautia vs. **body weight**	0.08	ns
g__Coprococcus 1 vs. **body weight**	-0.08	ns
g__Marvinbryantia vs. **body weight**	0.2	ns
g__[Eubacterium] ventriosum group vs. **body weight**	-0.02	ns
g__Ruminococcaceae UCG-005 vs. **body weight**	0.39	ns
g__Ruminococcus 1 vs. **body weight**	0.4	ns
g__[Eubacterium] coprostanoligenes group vs. **body weight**	0.01	ns

5.3.4 **Characterisation of the synovial fluid, urine, blood, and serum bacterial populations by 16S rRNA sequencing**

The characterisation of bacterial DNA in the synovial fluid, urine, blood, and serum was assessed by PCR amplification and sequencing of the bacterial 16S rRNA gene, variable region 4. Applying PCR amplification, bacterial 16S rRNA was absent in the synovial fluid of CIA and healthy controls mice.

In mouse urine samples, bacterial 16S rRNA was found in 90% (9/10) of CIA and 100% (10/10) of controls mice samples. Further, there was amplification in negative kit control (DNA extraction kit control).

In mouse blood samples, 16S rRNA was detected in 80% (8/10) of CIA and 90% (9/10) of controls mice samples. In mouse serum samples, 16S rRNA was found in all CIA and healthy controls samples. Moreover, a negative Kit control sample of DNA extraction kit for blood and serum produced a visible band during PCR steps.

5.3.5 **Bacterial Community Composition of urine**

At the phylum level, our urine samples were dominated by *Proteobacteria* (CIA= 58.8%, healthy controls=88.3%, and negative kit control= 92.7%), *Firmicutes* (CIA= 36.4%, healthy control=8.6%, and negative kit control= 4.8%).

At the species level, our urine samples were dominated by the species *Serratia sp. SBS* (CIA= 51.4%, healthy control=80.2%, and negative kit control= 81.1%, followed by species *Staphylococcus aureus* (CIA= 14.4%, healthy control=1.4%, and negative kit control=

0.6%).To a lesser extent, the urine samples contained species *bacterium N3a* (CIA= 2.2%, healthy control=3%, and negative kit control= 3.8%) **(See Figure 5-7).** Form these findings; we can see the source of common microbial taxa produced from the urine of CIA and Controls were from PureLink™ Microbiome DNA Purification Kit, which was contaminated with species *Serratia sp. SBS* and *bacterium N3a*. Further, *Staphylococcus aureus* was more present in CIA than controls, with no statistically significant (P-value = 0.075) between two groups, and also the abundance *Staphylococcus aureus* in bacterial taxa of kit negative control was 0.6%.

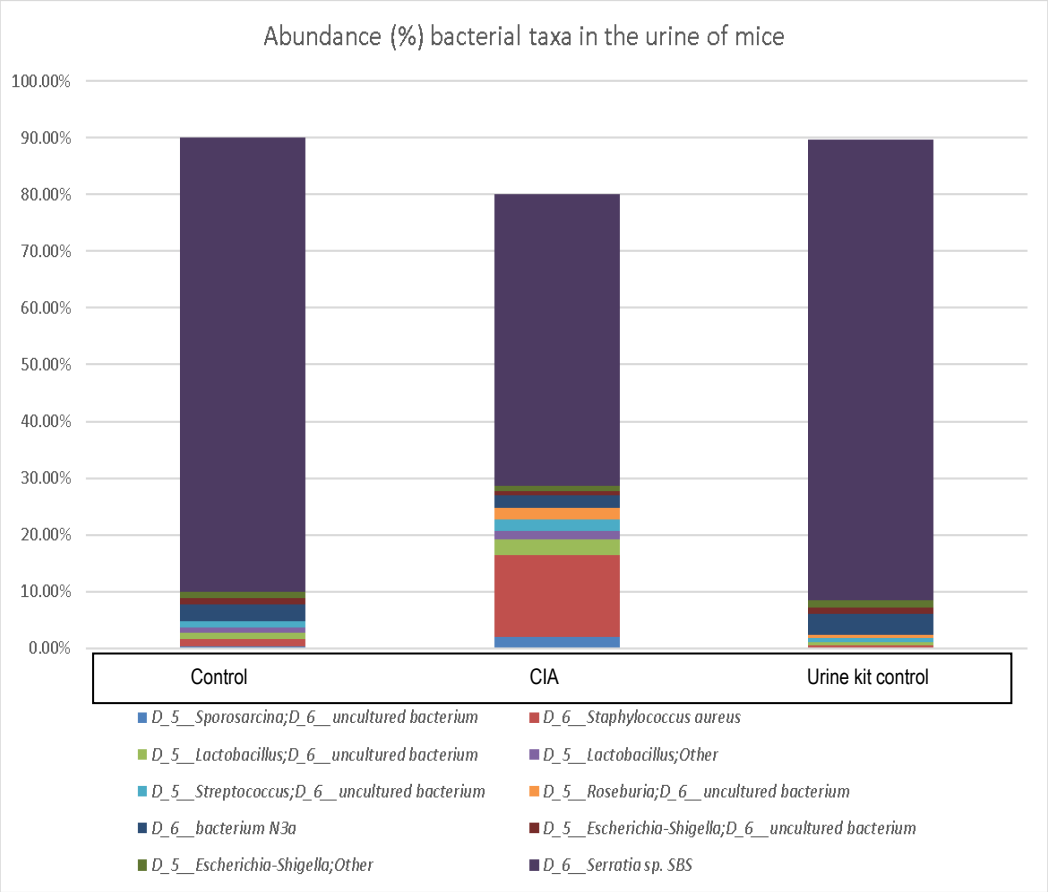


Figure 5-7 Relative abundance of bacterial taxa found within the urine mouse study. Data are the relative abundance of the most bacterial taxa, characterized as having a mean abundance of >1% of the total bacteria content in any one experimental group, detected in the urine of CIA, n = 9, healthy control, n = 10, and kit control, n =1 sample as determined using amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

5.3.6 **Bacterial Community Composition of blood**

At the phylum level, blood was identified to be dominated by members of the *Actinobacteria* (CIA= 29.5%, healthy control=35.4%, and negative kit control= 37.5%), *Firmicutes* (CIA= 30.5%, healthy control=30.7%, and negative kit control= 22.2%), *Proteobacteria* (CIA= 32.9%, healthy control=26.3%, and negative kit control= 35.2%) phyla.

At the genus level, our blood samples were predominated by the genus *Corynebacterium 1,* which accounted for 18.4%, 23%, and 24.8% of the total bacterial sequence detected in the CIA, healthy controls donors, and kit control respectively. Followed by the genera Streptococcus (CIA= 11.7%, healthy control=10.7%, and negative kit control= 7.3%), and *Serratia* (CIA= 11.4%, healthy control=10.9%, and negative kit control= 9.1%), *Propionibacterium* (CIA= 6%, healthy control = 6.9%, and negative kit control= 6.6%). To a lesser extent, the blood samples contained genera *Staphylococcus* (CIA= 3.5%, healthy control = 5.1%, and negative kit control= 3.1%), *Acinetobacter* (CIA= 4%, healthy control = 2.9%, and negative kit control= 10.5%), and *Enhydrobacter* (CIA= 2.5%, healthy control = 1.7%, and negative kit control= 1%) **(See Figure 5-8)**. Form these results; we can see the source of common microbial taxa produced from the blood of CIA and controls were from DNeasy® Blood and Tissue Kit, which was contaminated with some bacterial taxa such genera *Corynebacterium 1, Streptococcus, Serratia, Propionibacterium, Staphylococcus, Acinetobacter*, and *Enhydrobacter.*

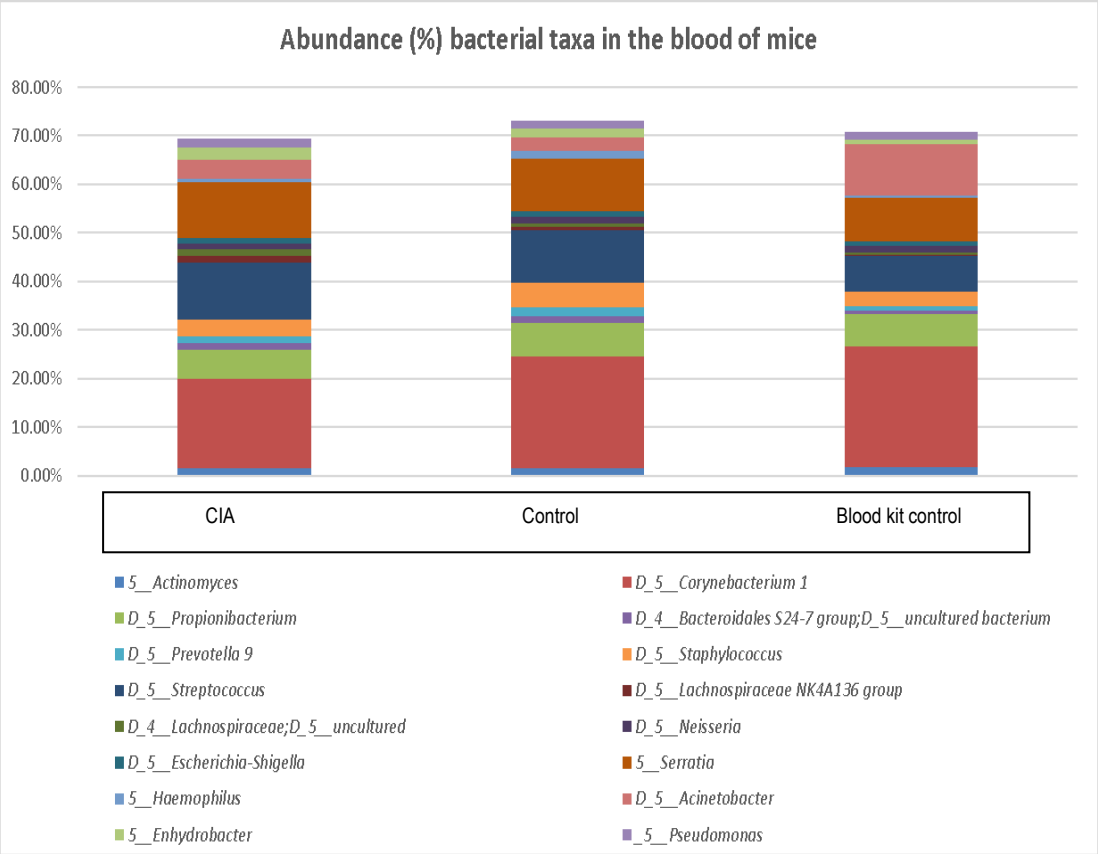


Figure 5-8 Relative abundance of bacterial taxa found within the blood CIA mouse. Data are the relative abundance of the most bacterial genera, characterized as having a mean abundance of >1% of the total bacteria content in any one experimental group, detected in the blood of CIA, n = 8, healthy control, n = 9, and kit control, n =1 sample as determined utilising amplification and sequencing of the 16S rRNA gene. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

5.3.7 **Bacterial Community Composition of serum**

At the phylum level, our serum samples were predominated by *Firmicutes* (30.7%), *Actinobacteria* (26.9%), *Bacteroidetes* (20.7%), and *Proteobacteria* (18.9%).

At the species level, our serum samples were predominated by an unidentified member of the *Bacteroidales S24-7 group* (CIA= 9.3%, healthy control= 26.5%, and negative kit control= 8.6%, and Corynebacterium 1 (CIA= 11.5%, healthy control=12.6%, and negative kit control= 13.6%). Followed by an unidentified member of the *Propionibacterium* (CIA= 9.6%%, healthy control=3.7%, and negative kit control= 6.3%) and the species *Serratia sp. SBS* (CIA= 8.5%, healthy control=4.1%, and negative kit control= 5.6%) **(See Figure 5-9).** Form these findings we can show the source of common microbial taxa generated from the blood of CIA and controls were from DNeasy® Blood and Tissue Kit, which was contaminated with some bacterial taxa such an unidentified member of *the Bacteroidales S24-7 group*, *Corynebacterium 1, Propionibacterium,* and species *Serratia sp. SBS.*

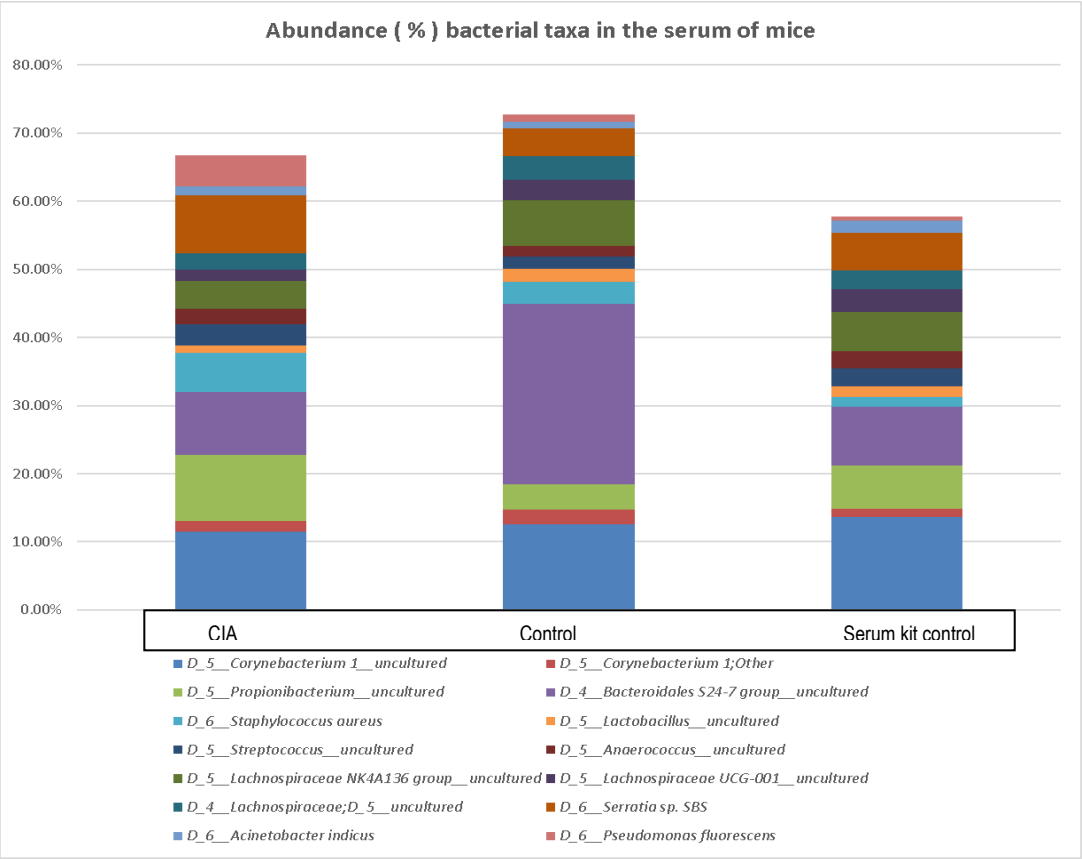


Figure 5-9 Relative abundance of bacterial taxa found within the serum mouse study. Data are the relative abundance of the most bacterial taxa, characterized as having a mean abundance of >1% of the total bacteria content in any one experimental group, observed in the serum of CIA, n = 10, healthy controls, n = 10, and kit control, n =1 sample as determined utilising amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

5.3.8 **Contaminated taxa in DNA extraction kits**

We provide an extensive list of possible contaminating taxa with expression levels >1% in DNA extraction kits in **Table 5-3**.

Table 5-3 List of contaminant taxa found in sequenced negative kits controls

DNA Extraction kits	List of constituent contaminant taxa
PureLink™ Microbiome DNA Purification Kit	**For urine** *D_6__bacterium N3a* *D_5__Escherichia-Shigella;D_6__uncultured bacterium* *D_5__Escherichia-Shigella;Other* *D_6__Serratia sp. SBS*
DNeasy® Blood and Tissue Kit	**For blood** *5__Actinomyces* *D_5__Corynebacterium 1* *D_5__Propionibacterium* *D_4__Bacteroidales S24-7 group;D_5__uncultured bacterium* *D_5__Prevotella 9* *D_5__Staphylococcus* *D_5__Streptococcus* *D_5__Lachnospiraceae NK4A136 group* *D_4__Lachnospiraceae;D_5__uncultured* *D_5__Neisseria* *D_5__Escherichia-Shigella* *D-5__Serratia* *5__Haemophilus* *D_5__Acinetobacter* *5__Enhydrobacter* *_5__Pseudomonas* **For serum** *D_5__Corynebacterium 1__uncultured bacterium* *D_5__Corynebacterium 1;Other* *D_5__Propionibacterium__uncultured bacterium* *D_4__Bacteroidales S24-7 group__uncultured bacterium* *D_6__Staphylococcus aureus* *D_5__Lactobacillus__uncultured bacterium* *D_5__Streptococcus__uncultured bacterium* *D_5__Anaerococcus__uncultured bacterium* *D_5__Lachnospiraceae NK4A136 group__uncultured bacterium* *D_5__Lachnospiraceae UCG-001__uncultured bacterium* *D_4__Lachnospiraceae;D_5__uncultured* *D_6__Serratia sp. SBS* *D_6__Acinetobacter indicus*

5.4 Discussion

5.4.1 Characterisation of the intestinal microbiome of mice

A growing body of evidence indicates that the intestinal microbiome has a role in the initiation and development of RA. Recently, mouse models have been utilised to investigate the interaction between host and microorganism in the intestinal microbiome, increasing the understanding of the pathological mechanism of RA and for the testing of new products for their anti-arthritic, which may help to improve arthritis condition (Liu *et al.*, 2016). Despite the availability of data associated gut microbiome dysbiosis to CIA, there is a need to assess if there is a consistency in data, or if factors such as differences in induction methods would lead to a difference in these. To this end, we characterised the stool microbiome of collagen-induced arthritis mice as compared with healthy control mice to confirm the gut dysbiosis, and then to endeavour in the same animals for signatures of these taxa in the blood, synovial fluid, and urine. This study was performed to link dysbiosis in multiple microbiome niches in the collagen-induced arthritis model following the induction of experimental RA.

. Pathophysiologic mechanisms via which intestinal microbiota contribute to the development of RA are complex; proposed mechanisms involve modifications in the permeability of gastrointestinal mucosal, stimulation of antigen-presenting cells via an impact on Toll-like receptors or NOD-like receptors, antigenic mimicry, the capability to generate citrullination of peptides via enzymatic action, development of T helper type 17-mediated mucosal inflammation, and regulation of host immune system (producing T cell differentiation) (Horta-Baas,2017).

At the phylum level, our stool samples were dominated via two key phyla; *Firmicutes* and *Bacteroidetes.* These findings agreed with the previous study by Liu and co-workers (Liu *et al.*, 2016) and further support the notion of a core stool microbiome of mice predominated by two key phyla.

We identified key taxa that appeared to be differentially present or abundant in mice with CIA or healthy controls. We found that the CIA mice were associated with significantly increased levels of genera *Acetatifactor, Blautia, and Coprococcus 1,* and *Marvinbryantia* when compared to those with healthy control mice. They are bacterial genera belonging to the family of *Lachnospiraceae*, which usually Inhabit in human and also mammalian gastrointestinal microbiota (Kanki and Grimes, 2012). It has been found that *Lachnospiraceae* can stimulate T-helper1 and T-helper17 polarisation and down-control T regulatory response (W. Wu *et al.*, 2016). Furthermore, members of *Lachnospiraceae* also can enhance pathogenesis (Krych, & Hansen, 2015). It is well established that Increased Lachnospiraceae was increased in the gut of the RA patients (X. Wu *et al.*, 2016).

Genus *Acetatifactor* was initially isolated from the gut of an obese mouse and maybe fed a high-fat diet (Lagkouvardos *et al.*, 2016).

Further, it has no deleterious effects on the normal abundance of *Blautia* in humans. However, several studies demonstrated that the abundance of genus *Blautia* was increased in many illnesses such as systemic lupus erythematosus, Crohn's disease, nonalcoholic fatty liver diseases, and irritable bowel syndrome (Qi *et al.*, 2016; Luo *et al.*, 2018). Moreover, the study by Liu et al. (Liu *et al.*, 2016) identified that there are differences in the intestinal microbiome composition between CIA-susceptible and CIA-resistant mice, with a predominance of genera *Acetatifactor*, *Blautia*, and *Coprococcus* in arthritis mice.

We also identified that the bacterial genus *Alistipes* was more abundant in the CIA mice than in healthy mice. The genus *Alistipes* has been implicated in the pathogenesis of diseases such as diabetes (Qin *et al.*, 2012), cancer (Fulbright, Ellermann and Arthur, 2017), and cardiovascular disease (Wang *et al.*, 2011). The associations are mediated via different pathways including affecting the mechanistic target of the rapamycin (mTOR) signalling pathway (mTOR is a kinase that regulates metabolism and cell growth in response to, growth factors, nutrients, and stress, and cellular energy) (Hall, 2008; Zheng *et al.*, 2017). Contrary to our finding, it was previously observed that the genus *Alistipes* was less abundant in CIA-susceptible mice prior to arthritis onset than in CIA-resistant mice (Liu *et al.*, 2016).

Eubacterium ventriosum group, Eubacterium coprostanoligens, Ruminococcus 1, and Ruminococcaceae UCG-005 were present in significantly lower proportions in CIA subjects compared with healthy control subjects. Eubacterium is negatively associated with inflammatory markers (van den Munckhof *et al.*, 2018). Further, *Eubacterium* is a beneficial microbe of gut bacteria, which involves several species that generate butyrate (Uematsu & Hoshino, 2003). Butyrate is observed as a substantial nutrient for intestinal epithelium cells, and it plays a fundamental role in normal development and the energy metabolism of these cells (Wong, & Jenkins, 2006). Further, butyrate has a function as an anti-inflammatory factor, through the inhibition of nuclear factor κB (NF-κB) activation in host colonic epithelial cells (Inan *et al.*, 2000), which might be an outcome from the inhibition of Histone Deacetylases. NF-κB controls several cellular genes implicated in early immune-inflammatory responses, such as IL-1b, IL-2, TNF-α, IL-6, cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), T cell receptor-α (TCR-α), vascular cellular adhesion molecule-1 (VCAM-1), and MHC class II molecule (Baeuerle and Henkel, 1994; Barnes and Karin, 1997; Jobin and Sartor, 2000).

Eubacterium ventriosum is more abundant in healthy populations of mice relative to colitic mice (Bibiloni, & Tannock, 2005). *Eubacterium coprostanoligens* is a cholesterol-reducing bacterium (Kriaa *et al.*, 2019). These bacteria alter cholesterol into coprostanol that is not absorbed through the gastrointestinal system of the host, thereby leads to lower levels of cholesterol. Several pieces of evidence indicate that links high cholesterol levels in the human body to increased risk of RA in women (KASAI *et al.*, 2016). We thus anticipate *Eubacterium* strains have several beneficial effects on human health and another prospective inhibitor of RA.

Here we also have been shown that genus *Ruminococcus* was decreased in CIA mice subjects compared to healthy control subjects. Several *Ruminococcus species,* except *R. gnavus*, are reduced in some autoimmune diseases such as Crohn's disease (Turesson *et al.*, 2015). Interestingly; several *Ruminococcus species* are mucin-degrading microbes and substantial in maintaining intestine homeostasis, in particular, through the production of Short-chain fatty acids (SCFAs). SCFAs, in turn, enhance intestinal health, generating a stable environment for resistance to the pathogen and also a defence against colitis (Joossens *et al.*, 2011). Our results in concordance with the recent studies, results of gut microbiome analysis, found that genus *Ruminococcus* was over-represented in healthy individuals when compared to those with RA and Psoriatic arthritis patients (Smith *et al.*, 2013; Wu *et al.*, 2017).

Genus *Ruminococcaceae UCG-005* also has been reported to be butyrate-generating bacteria that may protect healthy populations from chronic inflammation of the gut (Scher *et al.*, 2015). Leonardo et al. reported that genus *Ruminococcaceae UCG-005* was significantly associated with the gut of healthy subjects in comparison to ulcerative colitis (UC), pseudomembranous colitis (CDI) and Crohn's disease (CD) (Mancabelli *et al.*, 2017).Our results also pointed out that dysbiosis of CIA mice at terminal microbiome

measurements are not correlated with body weight factor. These results mirror previous studies (Peters *et al.*, 2018; Grembi *et al.*, 2020).

However, other studies found that the abundances of *Acetatifactor, Marvinbryantia*, and *Alistipes* were correlated with weight loss (Henning *et al.*, 2018; Liu *et al.*, 2019). However, the genera *Blautia* and *Coprococcus* were increased in overweight people (Castaner *et al.*, 2018). Further, *Eubacterium and Ruminococcus are* associated with a significant improvement in weight control and metabolic and inflammatory parameters (Clarke *et al.*, 2012; Abenavoli *et al.*, 2019). This also supports the hypothesis that the association between weight and RA is mediated via the gastrointestinal microbiome.The major limitation of the study is the significant weight loss in the CIA mice, which could be attributed to the loss of appetite and decrease of the physical activity of mice after the onset of symptoms of the disease(Kwon *et al.*, 2014). The change of weight in the CIA mice might impact on the microbiome composition in these animals during the induction of CIA.

It has been found that the CIA group had a specific decrease in fat mass and muscle weight, and CIA development related to increased lipolysis (Na *et al.*, 2017; Alabarse *et al.*, 2018). Besides, in adjuvant-induced arthritis animal model, it also has found decreased fat mass relative to control (Martín *et al.*, 2008; López-Menduiña *et al.*, 2010; Hamaguchi *et al.*, 2012; Alabarse *et al.*, 2018). Martín et al. (Martín *et al.*, 2008) propose that the mechanism is due to a reduction in white adipose mass are secondary to decreased adipose lipogenesis. Lopez-Menduina et al. (López-Menduiña *et al.*, 2010) have found decreased body weight gain in adjuvant-induced arthritis animals, proposing an inhibitory effect of inflammation on body weight, in which diseased animals have a lower relative fat mass when compared those with pair-fed healthy mice. Given that the modification in the bodyweight mass can alter the microbiome (John *et al.*, 2018).

This study indicated that the mouse intestinal microbiome of CIA subjects was different from that of healthy controls, evidenced by the changes in the genus taxonomic level, suggesting that the microbiome is associated with the CIA. Further studies are required to investigate whether the microbiome could be causative for RA. We suggest that characterised gut microbiome from the early stage, patients with RA, and CIA or other mouse arthritic models such as adjuvant arthritis model by 16s rRNA and ITS sequencing to identify the dysbiotic microbiome population in these subjects. Next, applying the faecal microbiota transplantation technique (the process of transplanting faecal bacteria from a donor into a recipient) to normalise the imbalance of intestinal microbiome community structure and examining whether this modification alters the disease process by measuring the disease severity and serum inflammatory cytokines such as IL-6, IL-17, IL-22, and IL-23.

5.4.2 **Characterisation of synovial fluid, blood, and serum microbiome of mice**

In this part, we attempted to characterise synovial fluid, urine, blood, and serum in CIA and healthy controls.

There was no bacterial DNA in all synovial fluid of CIA and healthy controls. Many properties of synovial fluid may specifically inhibit the detection of intra-articular bacteria via PCR amplification. Synovial fluids of animals may contain low bacterial DNA (Scharf *et al.*, 2015). Older studies have hypothesised that leucocyte DNA may inhibit the detection of small amounts of bacterial DNA with some PCR protocols (PALMER and BERTONE, 1994; Jordan and Durso, 2005; Bonilla *et al.*, 2011; Scharf *et al.*, 2015).

For the urine, blood, and serum of mice study, our findings presented here show that contamination with bacterial DNA or cells in negative kit control of DNA extraction kits, and that this contamination affects the results obtained from these the samples. Bacterial DNA contamination arising from DNA extraction kits may have an in particular massive impact when studying low-microbial biomass samples, for example, blood and urine, which may provide a low DNA for competing with it in reagents for amplification (Salter *et al.*, 2014a; Glassing *et al.*, 2016). Contaminating DNA has been reported from DNA extraction kits many times (Mohammadi *et al.*, 2005; Shen, Rogelj and Kieft, 2006; Salter *et al.*, 2014b; Glassing *et al.*, 2016; Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019). Whilst it is potential that the suspect taxa are genuinely existent in these samples, in several conditions, they are biologically unexpected, for instance, *Serratia* associated bacteria that have been involved in human illness (Khanna, Khanna and Aggarwal, 2013; Fine *et al.*, 2019; Gajdács *et al.*, 2019).

The bacterial taxa in **Table 5.3** have been previously reported as contaminated bacterial taxa in DNA extraction kits (Salter *et al.*, 2014a; Lauder *et al.*, 2016a; Stinson, Keelan and Payne, 2019; Weyrich *et al.*, 2019). These results have several limitations. Our findings do not rule out the presence of microbiome in urine, blood, and serum of mice samples, which must be an indication that we cannot utilise our results to differentiate urine, blood, and serum of mice samples from contamination kit controls.

For future studies, the recommendations for the use of urine, blood, and serum microbiome samples and low biomass samples are as follows:

1. Provided that it includes low biomass samples, at the beginning of the study, it is necessary to conduct some form of absolute abundance measure (here qPCR of

total copies of total 16S rRNA genes) to determine if samples of low biomass are in effect significant so that it is convenient. Precautions must be taken.

2. Contaminated kit controls must be produced and analysed contemporaneously with low biomass samples. This is because some of the DNA extraction kits are reported to have diverse contaminants, and contamination may vary depending on the batch (Salter *et al.*, 2014b; Weiss *et al.*, 2014; Lauder *et al.*, 2016b). Therefore, comparing unmatched samples and controls may result in erroneous differences.

3. When working with low biomass samples such as urine and blood, it is best, to begin, with the null hypothesis that all samples study is contaminated only and ask if this concept can be rejected with this data.

4. It would be useful when reviewing microbiome analysis; low biomass samples should be continuously requested so that authors report contamination controls; their techniques for rejecting the hypothesis that all samples have contamination only.

5. Post hoc analysis, where the parent groups found no global difference, can be risky. Ideally, the results of any such analysis will be re-examined in an independent validation cohort.

5.5 **Conclusion**

This study indicated that the mouse intestinal microbiome of CIA subjects was different from that of healthy controls in agreement with previous animals and human studies. Our findings demonstrated that some changes in the gut microbiome are associated with RA, evidenced by the changes in the genus taxonomic level.

Chapter 6

6 Research discussion and future work

This chapter provides a general discussion of the findings of our research and discusses the novelty of the research in more detail. Here, I discuss my findings, from two perspectives; (1) Dysbiosis in blood and synovial fluid of RA patients and (2) Characterisation of stool, urine, synovial fluid, blood, and serum of mice with CIA and healthy controls. The following figure summarises what we have done to achieve our aims.

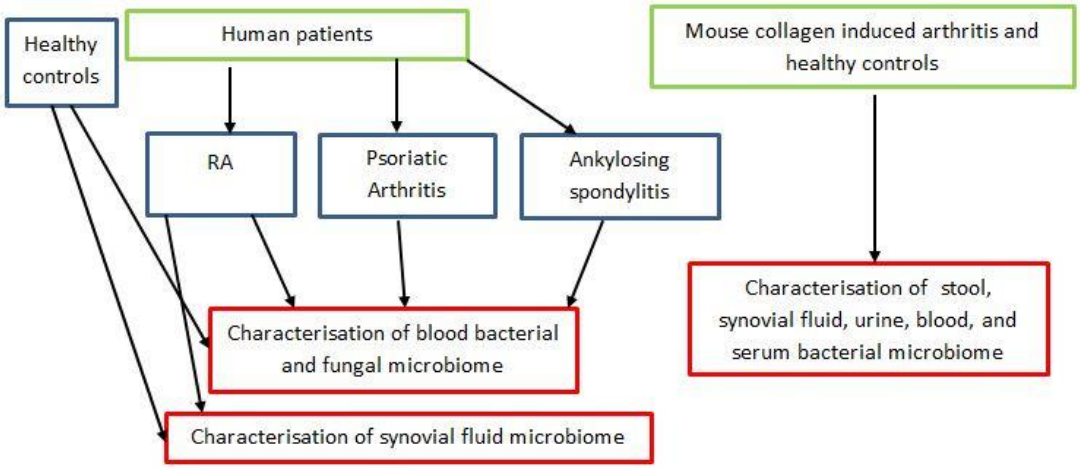


Figure 6-1 A flow chart summarising our research

6.1 Dysbiosis in blood and synovial fluid of RA patients

There is evidently a gap in research in the uncovering of how blood dysbiosis is associated with the development of Rheumatoid Arthritis (RA), Ankylosing Spondylitis (AS), and Psoriatic Arthritis (PA), as no data is available in the literature about this relation. This directed our studies and governed the steps taken in this research, permitting the investigation and development of a diagnostic tool for a pool of candidate biomarkers. The purpose was to understand the pathogenesis of RA.

In this research, I have characterised the blood microbiome of patients with RA, AS, and PA, in comparison with healthy control subjects. I have also attempted to identify specific microbiome signatures to these illnesses and determined the correlation of inflammatory cytokines (IL-17-A, IL-22, IL23, and IL-6) levels with RA disease. Furthermore, I have attempted to show how the microbiome changes post-treatment of RA patients. One of the main results of the study shows that that out of 17 RA patients, 76.47% (13 patients) progressed towards the healthy microbiome after induction of treatment. This, in turn, reveals that dysbiosis is associated with RA. In addition, the results also showed a better normalisation of the serum microbiome among the seronegative RA cohort. While several studies do not differentiate between treatment response and seropositivity, a recent study showed that seronegative RA might show a better response to treatment (Choi and Lee, 2018), supporting our findings.

Another finding of significance is mentioned in **Table 3-1**. Here, all the microbiome alterations of RA patients indicate the occurrence of dysbiosis. The results here revealed that the genera *Halomonas* and *Shewanella* were significantly increased in the serum of RA patients (RA V0 and RA V3) relative to healthy controls. This finding suggests

thatRA has been associated with dysbiosis of the blood microbiome . This is a novel study on the relation of *Halomonas* and *Shewanella* presence in blood and RA pathogenesis.

Further*, Achromobacter, Escherichia/Shigella*, Serratia*, Corynebacterium-1, Streptococcus, Granulicatella, Staphylococcus,* and *Gemella* were the genera significantly decreased in abundance in the serum of RA patients relative to control subjects. Therefore, their decrease, as we have observed in the blood microbiome in serum RA patients, along with the other alterations mentioned above, indicates the occurrence of dysbiosis in these distant locations.

In considering the fungal microbiome, the fungal ITS2 gene was not seen in the serum of RA patients (RA V0 and RA V3). However, fungal ITS2 was detected in the serum of patients with AS, plasma with RA, serum, and plasma with PA, and plasma and serum of healthy control subjects. We found no statistically significant differences in the fungal blood population between diseased groups and healthy control subjects.

The fundamental role of cytokines in the pathophysiology of RA was evident as there was a notable elevation of cytokines, IL-6, IL-17, IL-22, and IL-23 levels in the serum and plasma of RA patient, in comparison with healthy control. This indicated and supported the idea that such cytokines are associated with joint destruction (Srirangan and E. H. Choy, 2010; Yoshida and Tanaka, 2014a; Narazaki, Tanaka and Kishimoto, 2017; Boyapati et al., 2019).

With respect to the association between RA and bacterial dysbiosis of synovial fluid, there has been very little research reported (Zhao et al., 2018), and to date, no data is available about fungal dysbiosis in the synovial fluid of RA patients. This provided us with the stepping platform to investigate the presence of bacterial and fungal DNA (by 16S and ITS2 respectively) in the synovial fluid of human RA patients in comparison to the healthy

control, using subjects utilising next-generation sequencing and bioinformatic techniques. In addition, we also compared the differences of the bacterial and fungal microbiome in synovial space with the level of selected cytokines in the synovial fluid.

Our findings showed that *Raoultella* (p = 0.051) was more abundant in RA synovial fluid compared to healthy subjects. Two dominant species are able of causing infections in human: *Raoultella ornithinolytica* and *Raoultella planticola*, and are capable of causing infective arthritis (Seng et al., 2016; Venus et al., 2016; Bonnet et al., 2017; Levorova et al., 2017). It has been observed that *Raoultella* is associated with gut infections, and the possible cause of such infection was considered to be enteric translocation (Campos, Guimarães and Lovisolo, 2016). *Raoultella* genus may gain access to the synovial fluid from the above-mentioned infection locations, and it may also be responsible for the triggering of autoimmune responses and initiating RA.

For fungal populations, *Hypocreales* (P = 0.012), and *Aspergillus* (P = 0.14 including all data, P = 0.057 excluding samples where <1% of reads mapped to this taxa) were more abundant in RA synovial fluid relative to healthy subjects. *Hypocreales* order and genus *Aspergillus* has been detected in different anatomical sites of the human body including the intestinal tract, oral cavity, and blood (Peters et al., 2017a, Beatty *et al.*, 2014; Panaiotov et al., 2018, Ghannoum *et al.*, 2010; Hallen-Adams and Suhr, 2017). These taxa may potentially reach the synovial fluid from these places and contribute to the pathogenesis of RA.

Unclassified organisms belonging to the order *Malasseziales* (P = 0.002) and genus *Cladosporium* (P = 0.019) were less abundant of RA synovial fluid compared to healthy subjects. *Malasseziales* was found to vary in abundance in the case of inflammatory bowel disorder (Sokol *et al.*, 2017; Witherden *et al.*, 2017); suggesting it may be responsive to an

inflammatory environment. *Cladosporium* has been identified in the healthy human gut (Hallen-Adams and Suhr, 2017), and oral (Peters et al., 2017b), and is encountered commonly in human clinical samples (Sandoval-Denis *et al.*, 2015). Further, gender and age could be co-influence factors on microbiome population in RA synovial fluid.

Our results also provide evidence for the presence of bacterial and fungal DNA in classically sterile areas such as the blood and synovial fluid, leaving the possibility of:

1) Microbial translocation from classical niches (e.g., the gut and oral) into the bloodstream, a process termed atopobiosis, followed by killing these microorganisms by an immune cell known as a phagocyte leaving only the DNA.
2) The microbial nucleic acid may reach the synovial place through the blood (as evidenced by the simultaneous presence in both fluids).
3) Microbial translocation followed by a state of dormancy due to activation of the immune system or to unfavourable environmental conditions

With relevance to the primary two approaches, it is well confirmed that the immune system could distinguish self from non-self-nucleic acid (DNA and RNA) in the human blood and synovial fluid via specific pattern recognition receptions (Chi and Flavell, 2008) and that TLR9 recognises foreign DNA, and RNA is identified via TLR3 on immune cells, producing in the upregulation of various pro-inflammatory cytokines such as TNF *alpha, and IL-6,* which are associated with *RA* (Atianand and Fitzgerald, 2013; Castañeda-Delgado *et al.*, 2017). With importance to the latter scenario, dormant microorganism's cells may colonise the joints of RA patients, unidentified by routine culture, while maintaining the capability to stimulate an immune system by their lipopolysaccharide (LPS) and other antigenic components (Pretorius *et al.*, 2017). It showed that bacterial LPS could activate many of the common RA-associated cytokines, for instance, TNFα, the IL-1 family, IL-6, IL-12 family, and IL-15 (Rossol *et al.*, 2011; Li *et al.*, 2014).

Microbial translocation can be investigated by culture-independent technology**,** culture blood, and detection of LPS:

A- **By culture-independent technology such as 16S rRNA and ITS genes sequencing-** the translocation of microbial DNA from classical niches into the bloodstream could be examined in human by performing 16S rRNA and ITS2 sequencing on samples obtained from different body habitats for the same subjects, for example, the gut and oral with blood to identify origin the microbial DNA, which translocate into the blood. Further, the translocation of microbial DNA from classical niches into the blood could be investigated in mice by colonising known bacterial cultures into different body sites of the GF mice, for example, the gut, and oral and then applying 16S rRNA and ITS2 sequencing on samples obtained from different body habitats to determine which environments the known microbiota translocate to the following inoculation.

Advantages of culture-independent technology:

1- Culture-independent technology provides extensive and in-depth information about microbial communities on different sites of the human body.

2- Allows investigation of information about microbial populations without culturing (Jo, Kennedy and Kong, 2016)

Limitations:

Although 16S rRNA and ITS genes sequencing are a potent tool to understand how the blood microbiome population is associated with RA, there are limitations. Generation of Chimera and the intrinsic error rate of sequencing are significant considerations (Jo, Kennedy and Kong, 2016). A chimera which is an artifact produced through the PCR process is a single sequence consisting of fragments from two or more different

sections(Jo, Kennedy and Kong, 2016). In later PCR cycles, Chimeras are thought to result when prematurely terminated amplicons are utilised as primers (Jo, Kennedy and Kong, 2016). Both chimera and sequencing errors occur in nonsense sequences that may be incorrectly recognised as new species or incorrectly classified (Jo, Kennedy and Kong, 2016). To order to address these technological limitations, algorithms that recognise and delete chimera sequences (e.g. UCHIME) and sequencing errors (e.g. denoising) have been used (Jo, Kennedy and Kong, 2016).

Yet another important challenge is distinguishing cause-and-effect associations. 16S rRNA and ITS genes sequencing capture microbial profiles at a specific moment in time (Jo, Kennedy and Kong, 2016). It is therefore difficult to determine that the changing microbial population caused the disease, or resulted from it. Thus, further hypothesis-testing mechanistic examinations are needed (Jo, Kennedy and Kong, 2016).

Further, 16S rRNA and ITS genes sequence result are relative rather than absolute, such that the actual quantity of a specific microbiome is unknown(Jo, Kennedy and Kong, 2016). While more direct and comprehensive than culture-based approaches, 16S rRNA and ITS genes sequencing also has biases: each 16S rRNA and ITS genes may not amplify with equal efficiency through PCR reactions due to differential primer affinity and GC content (Jo, Kennedy and Kong, 2016). Besides, taxonomy assignment is reliant on the completeness of reference databases. The quality and quantity of references define the accuracy and resolution of the taxonomic classification, and outcomes may differ depending upon the choice of the reference database (Jo, Kennedy and Kong, 2016).

B- By using blood culture- the evidence of microbial translocation is also by the blood culture includes the detection of entire microbiota in cultures of the portal or peripheral blood from other distal niches such as the gut (De Madaria *et al.*,

2005). Aerobic and anaerobic blood culture bottles are continuously incubated at 37°C for 5 days with 10ml of blood in BACTEC culture vials. All blood cultures with positive readings are additionally cultured into chocolate blood agar, Columbia blood agar, and CLED media. Isolates are detected by standard microbiological tests (De Madaria *et al.*, 2005).

Advantages of blood culture:

Evaluated favourably in detecting growth, it is useful in small laboratories with a small workload, and cost-effective (William, 2012).

Limitations:

More false positives, the lower yield of anaerobes, and labour intensive required to inspect for growth visibly (William, 2012).

C- By detection of LPS: lipopolysaccharide or Endotoxinis an essential and integral component of the external membrane of all Gram-negative bacteria and that induces bacterial translocation, for example, from the gut (Vaishnavi, 2013). Portal hypertension leads to intestinal sub-mucosal oedema that obstructs the protective integrity of the mucosal barrier and appears in an imbalance of the gut microflora, increased bacterial endotoxin-mediated mucosal injury and impaired mucosal defences. Detection of endotoxin from the blood is also a method to identify bacterial translocation (Vaishnavi, 2013). The Limulus amoebocyte lysate assay identifies endotoxins in body fluids. LPS causes the clotting of extracts of amoebocytes of the horseshoe crab, Limulus polyphemus (Vaishnavi, 2013). Non-specific amidase and other inhibitors in human plasma, while, interferes with the results. Recently, two new quantitative endotoxin microplate assays

applying homogenous and heterogenous fluorescence phage recombinant technology and recombinant horseshoe crab factor C that eliminates sample inhibitory effects have become available (Vaishnavi, 2013).

It would be interesting considering that *Halomonas* and *Shewanella* in the blood and *Raoultella* and *Hypocreales* in the synovial fluid are associated with RA. Furthermore, the composition of the microbiome such as *Achromobacter, Corynebacterium-1, Streptococcus, and Gemella* in the blood, and *Malasseziales and Cladosporium* in the synovial place may also have a protective role against the development of autoimmune conditions, as proposed by the presence of these microbial DNA from healthy individuals more than RA patients, for this, we hypothesised that differentially abundant microbiome genera in blood and synovial fluid could be used as markers for RA diagnosis.

Regarding the cytokines investigated, the results here observed higher concentrations of IL6, IL-17-A, IL-22, and IL23 in the synovial fluid of RA patients relative to healthy control subjects. The data revealed by a number of authors in the literature agreed with our results (da ROCHA et al., 2012; Elhewala et al., 2015; Yago et al., 2017; Boyapati et al., 2019), showing further support to our findings. When looking into a detailed synthesis of our findings, we found that an IL-6 level was higher among a sub-cluster of the RA cohort defined according to the fungal and bacterial microbiome. This is supported by the fact that fungal pathogen is known to stimulate IL-6 expression (Narazaki, Tanaka and Kishimoto, 2017b). Furthermore, Th17 cells are essential in the immune response against fungal pathogens, and IL-6 is implicated in the differentiation of lymphocytes to Th17 cells (Srirangan and D. E. H. Choy, 2010). This suggests that the variation in the microbiome

within RA patients might be associated with illness severity by an IL-6 – Th17 mediated pathway.

In conclusion, we have described the characterisation of blood microbiomes of RA, AS and PA patients, compared to healthy control subjects, and examined the blood microbiomes as novel biomarkers for the pathogenesis of these illnesses. We identified specific microbiome signatures and determined the correlation of inflammatory cytokines relative to these diseases. Using precise and accurate methods of molecular techniques, including 16S rRNA and ITS2 sequencing, the investigation of the present composition of bacterial and fungal DNA in the blood samples was possible. The novelty lies in the work we have done here on microbial DNA in the blood, in comparison to the more studied variation in the gut, oral, lung and urinary tract of RA patients. Furthermore, we have shown an increase in cytokines, such as IL-6 in the serum, in patients with arthritis conditions. What is interesting in this study is how results varied between the beginning of the diagnosis and after the induction of the microbiome and how it partly normalises the treatment of patients with RA. Furthermore, we described the characterisation of the synovial fluid microbiome of RA in comparison to healthy control subjects. This is also a relatively new concept and focusing on the fluid itself of both bacterial and fungal microbiome.

6.2 Characterisation of stool, urine, synovial fluid, blood and serum of CIA and healthy control

In this study, we worked with mice to enable the characterisation of stool, urine, blood and serum. This takes advantage of the similarity between mouse and human in physiology

and anatomical structures and provides a valid reason as to why mouse models have been widely utilised in biomedical studies. The advantages of mouse modelling are vast. Primarily, the intestinal tracts in both mice and humans are composed of anatomically similar organs (Hugenholtz and de Vos, 2018). Secondly, mice have a low maintenance cost, relative to other mammalian experimental models. Thirdly, mice have a high reproductive are and a short life cycle (Nguyen *et al*., 2015).

In stool microbiome composition, the exception of a higher abundance of *Actinobacteria* in human, the phylum level microbiome population showed to be similar in mice and human, although the *Firmicutes*: *Bacteroide*s ratio was slightly higher in human (Nagpal, Wang, *et al.*, 2018). Such differences in the *Firmicutes*: *Bacteriodetes* ratio between human and mice could imply a limitation to the utilisation of these animal models since the communities of significant groups belonging to these phyla might not reflect the ratio typically observed in the gut of a healthy human. Nevertheless, these models may still provide a valuable area of study of human gut-related maladies, showing that the proportions of *Firmicutes* are usually found to be changed in patients with inflammatory conditions (Nagpal, Wang, *et al.*, 2018).

The characterisation of the current available RA models is required here with Collagen-induced arthritis (CIA) being on a prevalent option. CIA can be induced in DBA1 mice via high-quality collagen. CIA shares several similarities pathological and immunological features with human RA and has been extensively studied to explore pathogenesis as well as test candidates treatments (Brand, Latham and Rosloniec, 2007b). There is an increasing appreciation of the role of the intestinal microbiome dysbiosis in the evolution of RA (Quanqiu Wang and Xu, 2019). Utilising the CIA mice model, we characterised the gut, blood, synovial fluid and urine microbiome of collagen-induced arthritis mice relative to

healthy control mice to link dysbiosis in multiple microbiome niches in the collagen-induced arthritis model following the induction of experimental RA.

Our findings revealed that *the* genera *Acetatifactor, Blautia, and Coprococcus 1, Marvinbryantia were* more abundant in the CIA in comparison to healthy subjects. They are bacterial genera belonging to the family of *Lachnospiraceae*, which has been seen this taxon activate T-helper1 and T-helper17 polarisation and inhibit T regulatory reaction (W. Wu *et al.*, 2016). It is well established that Increased *Lachnospiraceae* has previously been described in RA patients with different disease duration (X. Wu *et al.*, 2016). *Alistipes* has been associated with the pathogenesis of autoimmune illnesses such as diabetes (Qin *et al.*, 2012).

The genera *Eubacterium ventriosum group, Eubacterium coprostanoligens, Ruminococcus 1, and Ruminococcaceae UCG-005 were* more abundant of the CIA relative to healthy subjects. *Eubacterium ventriosum* has been indicated to be more prevalent in healthy mice compared to colitic mice (Bibiloni, & Tannock, 2005). *Eubacterium coprostanoligens* is a cholesterol-reducing bacterium (Kriaa *et al.*, 2019). Much research indicated that links high cholesterol levels in the human body to increased risk of RA in human (KASAI *et al.*, 2016). The genera *Ruminococcus and Ruminococcaceae UCG-005* have been reported to be significantly associated with intestinal tract of healthy subjects in comparison to different autoimmune conditions, for example, RA, Psoriatic arthritis, ulcerative colitis (UC), pseudomembranous colitis (CDI) and Crohn's disease (CD) (Smith *et al.*, 2013; Mancabelli *et al.*, 2017; Wu *et al.*, 2017).

We also attempted to characterise synovial fluid, urine, blood, and serum in CIA and healthy controls. There was no bacterial DNA in all synovial fluid samples. The synovial fluid may contain a low number of bacteria and inhibitors substances such us leucocyte

DNA, which may inhibit DNA amplification by PCR (Palmer and bertonge, 1994; Jordan and Durso, 2005; Bonilla *et al.*, 2011; Scharf *et al.*, 2015). Our results presented here show that contamination with bacterial DNA in urine, blood and serum of mouse. Unfortunately, there was a contamination in the negative control of DNA extraction kits. Contaminating DNA has been found in DNA extraction kits several times by many researchers (Mohammadi *et al.*, 2005; Shen, Rogelj and Kieft, 2006; Salter *et al.*, 2014b; Glassing *et al.*, 2016; Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019). The bacterial taxa in **Table 5.3** have been previously identified as contaminated bacterial taxa in DNA extraction kits (Salter *et al.*, 2014a; Lauder *et al.*, 2016a; Stinson, Keelan and Payne, 2019; Weyrich *et al.*, 2019).

This contamination has affected our results; the sequence of bacterial DNA found in the test samples was identical with the sequence of DNA found in negative controls. We could not confirm whether there was a bacterial DNA in the tested samples, or it came from the contaminated kit. If these experiments would work, we could compare the outcome with what we found in human blood and synovial fluid to confirm that dysbiosis could be associated with RA.

6.3 Future work

In this work, a comprehensive study of the microbiome dysbiosis in RA human and animal has been presented. These data could be further expanded upon through a series of additional studies.

Further work should include in chapter 3 using a larger cohort of control and RA subjects to determine whether the microbiome biomarkers found would remain significant in a larger group of subjects, thus increasing the validity of the suggested blood microbiome biomarkers.

Moreover, it would also be useful to take blood samples from the same source. The different locations of donors might lead to the different microbiome patterns seen (Manasson, Blank and Scher, 2020).

Additionally, study the influence of characteristics of the patient population such as age, sex, and diet on the blood microbiome (Manasson, Blank and Scher, 2020). These factors may likely influence indirectly on the microbiota composition of the circulatory. As this study was the first study to determine if a circulatory microbiome could be detected and characterised from blood samples, possible, these factors were not taken into account.

Besides, our thought that the blood microbiome originates from one of the classical microbiome niches (the gut, mouth, urogenital tract, skin) and reaches the blood. However, in our study, only blood samples were obtained from the donors, and thus direct comparisons of the composition of the microbiome found in the different body places were not performed. In prospective studies, it would be valuable to take samples from various body sites such as gut, skin, oral, lung, with blood from the same donors to make comparisons of the microbiome at different body sites more useful to predict the likely source of the blood microbiome.

The culture technique will be used to detect the live microorganisms from distal niches such as the gut or oral microbiota into the blood. The portal or peripheral blood will be cultured. Aerobic and anaerobic blood culture bottles are continuously incubated at 37°C for 5 days with 10ml of blood in BACTEC culture vials. All blood cultures with positive readings are additionally cultured into chocolate blood agar, Columbia blood agar, and CLED media. Isolates are detected by standard microbiological tests (De Madaria *et al.*, 2005).

For further work should include in chapter 4, it would also be useful to take the blood samples with synovial fluid samples from the same patients to able compare synovial fluid profiles with blood to evaluate the effects of microbial dysbiosis at the blood on the synovial fluid microbiome.

Further, the culture technique will be used to detect the entire microorganisms in synovial fluid samples, as seen above.

In additional for chapter 5, we can use different approaches to extract DNA from various biological samples (blood, synovial fluid, and urine) from animal models such as using a Magnetic Bead DNA Isolation kit or even amplify 16s RNA amplicons using direct PCR with different systems, for example, a microFLOQ® swabs to avoid the source of contaminants, which has previously been seen with unconventional DNA extraction that affects all downstream applications (Ambers *et al.*, 2018; Manasson, Blank and Scher, 2020).

Our data support that there is an association of autoimmune disorder, particularly RA state, with microbiome dysbiosis in blood/tissues. Therefore, microbiome dysbiosis for autoimmune conditions offers potential opportunities for novel biomarker and therapeutic developments.

A number of studies and approaches will be required to confirm the cause and effects of the human microbiome on the development of RA.

It will be useful to characterise gut microbiome from the early stage, patients with RA, and CIA or other mouse arthritic models such as the SCW model by 16s rRNA and ITS sequencing to identify the dysbiotic microbiome population in these subjects. Next, applying the faecal microbiota transplantation technique to normalise the imbalance of the intestinal microbiome community structure and examining whether this modification alters the disease process by measuring the disease severity and serum inflammatory cytokines such as IL-6, IL-17, IL-22, and IL-23. Further, the Germ-free mice model provides an excellent framework to explore the cause and effects of the human microbiome on the development of RA (Kostic, Howitt and Garrett, 2013). GF mice are the most commonly used animal models to study host-microbe interactions. GF mice are raised in sterile conditions, have no microorganisms living in them, and can be made gnotobiotic by colonisation with single microbial species, allowing the impact of specific organisms to be studied (Kostic, Howitt, and Garrett, 2013). It would be useful to apply the faecal microbiota transplantation from patients with RA, whose microbiome composition was confirmed by 16S and ITS sequencing technique to germ-free mice. Then the mice will be evaluated for histological RA severity, and synovitis, gut permeability, and systemic inflammation.

Moreover, it would be useful to investigate the inflammatory properties of microbiome taxa, which have identified increase in RA illness such as by culture theses microbiome with some immune cells such as dendritic and measure their inflammatory receptors would prove very interesting, particularly in conjunction with detailed RA pathogenesis investigation.

The rheumatology field has produced development with the potential to significantly affect the care of our patients. One instance is the developing field of pharmacomicrobiomics, which explains the effects that microbial modifications have on the action and toxicity of drugs (and vice versa) (Rizkallah, Saad and Aziz, 2012). For example, it has understood that the activation of sulfasalazine, a disease-modifying antirheumatic drug applied to treat inflammatory arthritis conditions, is reliant on the enzymatic cleavage via intestinal microbiota (Peppercorn and Goldman, 1972). This seems to further hold for methotrexate, which is understood to be metabolised through the gut microbiota in humans and mice (Valerino *et al.*, 1972; Scher *et al.*, 2020) and may produce off-target antibiotic outcomes (Nayak *et al.*, 2019).

In axial SpA, patients that respond to anti-TNF inhibitors show a more resilient pretreatment intestinal microbiome (Bazin *et al.*, 2018); however, IL-17A inhibitors are associated with the expansion of gut C. albicans in a subgroup of patients with PsA/SpA (Manasson *et al.*, 2020), and an increased the risk for or invasive *candidiasis* (Mease *et al.*, 2019).

Additional development in pharmacomicrobiomics will lead towards personalised therapeutic strategies that are depended on patient microbiome characteristics, permitting for enhanced selection of medicines with the highest efficacy and lowest risk for toxicity (Scher *et al.*, 2020).

A different promising area of the current research is the research of targeted modulation of the microbiome to promote disease results, with the caveat that for most diseases, it is not yet clear whether microbial alterations contribute to illness pathogenesis or originate from the disease process itself.

It would be helpful to use the CIA model to show the effect of the microbiome in modulating RA progression. 16S rRNA and ITS sequencing will be performed to characterise the intestinal microbiome of DBA1 mice that will or will not produce RA after induction with collagen. After that, to determine whether the microbiome participates directly to RA progression, germ-free mice will be split into two groups, and one group will be colonised with microbiota from CIA-susceptible, and the other group will be colonised with microbiota from CIA- resistant mice by using faecal transplantation technique and will analyse the microbial community structures in each group. Following colonisation, mice will be induced with collagen II under germ-free conditions, as previously described in **chapter 2, 2.3**. Arthritic joints score will be examined by histopathology in germ-free mice after experimental RA in two groups to see arthritis incidence and severity in each group.

One indirect way to change the microbiome is through diet, which can globally shape the microbial population composition. Nutrition may change RA symptoms by increasing antioxidant levels, affecting the patient's metabolic profile, but also by changing the microbiota of the intestine. The gut microbiome can shift rapidly to dietary perturbations (Derrien and Veiga, 2017). Further, the gut microbiome is also included in the metabolism of some dietary ingredients and has the potential to alter circulating pro- or anti-inflammatory mediators (Schroeder and Bäckhed, 2016). For instance, trimethylamine-*N*-oxide, a pro-inflammatory metabolite that originates from carnitine and choline present in eggs, and red meat, is generated by *Prevotella copri* among other bacteria (Koeth *et al.*, 2013). An increased abundance of *Prevotella copri* was observed in new-onset untreated RA patients proposing *P. copri* may be pathogenic (Scher *et al.*, 2013). Dietary fibre, other complex carbohydrates, and sugar alcohols found in fruits are prebiotics and might also be beneficial via promoting a healthy microbiome (Lyte *et al.*, 2016). For example, microbial degradation of whole-grain complex carbohydrates increases short-chain fatty acids, which

were determined to be helpful to intestinal immune response (Bach Knudsen, 2015). Moreover, several foods have been identified as pro-inflammatory, including highly refined flours, gluten, dairy products (such as cheeses and milk), and red meat (Bustamante *et al.*, 2020). Some vegetables, such as eggplants, potatoes, and tomatoes, contain solanine, a glycoalkaloid, which was proposed to increase intestinal permeability and be detrimental for arthritogenic pathologies(Bustamante *et al.*, 2020).

On the other hand, other diets have been proposed to offer numerous health benefits, including long-chain omega-3 polyunsaturated FA (chia seeds, flaxseeds, fatty fish), monounsaturated FA (MUFA) (avocado, sesame), antioxidants, phytochemicals, flavonoids, vitamin D, fruits with enzymatic proteins such as black pepper and ginger (Bustamante *et al.*, 2020). Few conclusive studies exist on the topic, but some trials looking at the Mediterranean and vegetarian diets have determined beneficial effects in RA (Kjeldsen-Kragh *et al.*, 1991; Sköldstam, Hagfors, and Johansson, 2003). One of the investigations revealed that a diet trial with a Mediterranean diet presented a reduction in disease activity (DAS28) of 0.56 ($p < 0.001$) and in quality of life relative to the control diet (Sköldstam, Hagfors and Johansson, 2003). Fasting first and then eating a vegetarian diet for one year was helpful in RA, especially in terms of several swollen joints, stiffness, and CRP (Kjeldsen-Kragh *et al.*, 1991).

Obesity is associated with increased arthritis pain; weight loss may help improve arthritis symptoms. In psoriatic arthritis, which is strongly associated with metabolic syndrome and obesity, weight loss has also led to significant improvements in disease outcomes (Di Minno *et al.*, 2014; Klingberg *et al.*, 2019). The key to efficient weight loss may be to match nutrition and intestinal microbiota since recent investigations have observed that individuals with high Prevotella abundances in their intestinal microbiota lose more weight

on diets rich in fibre than people with low Prevotella abundances (Christensen *et al.*, 2019). These results also support the link between dietary fibre intake and *Prevotella* abundances, further emphasising microbial enterotypes as likely biomarkers in the personalised diet for obesity management.

Another approach relies on the use of probiotics, compounds that contain considered advantageous living organisms, and prebiotics, compounds that encourage the growth of beneficial microbes such as SCFAs (Bustamante *et al.*, 2020). Probiotics containing Lactobacillus have been associated with ameliorations in RA condition activity score (Hatakka *et al.*, 2003; Akkasheh *et al.*, 2016; Asquith *et al.*, 2017); however, Prebiotics have shown value in an animal model of SpA (Alipour *et al.*, 2014).

Another potential strategy, yoghurt contains a high number of types of beneficial bacteria such as *Bifidobacterium and Lactobacillus*, which may restore the dysbiosis in the gut (Bustamante *et al.*, 2020). Both gut microbial species produce a variety of tryptophan catabolites, which are critical for intestinal homeostasis by decreasing intestinal permeability (Roager and Licht, 2018). Therefore, it could be a supportive treatment for RA.

Several studies are indicating the positive effects of yoghurt consumption on human health. In particular, frequent consumption of yoghurt intake has been revealed to improve risk factors to lower the risk for dysbiosis and chronic kidney disease (Yacoub *et al.*, 2016), to lower diabetes risk (Margolis *et al.*, 2011), for cardiovascular disease (Ivey *et al.*, 2011), and enhance the development of host immunity (Daliri and Lee, 2015). For example, research on the effects of *Lactobacilli*-containing yoghurt on the composition of gut microbiota of healthy people who ingested a daily course for twenty days showed modifications for two groups of bacteria (the *C. coccoides-E. rectalei group, and*

Bacteroides and Prevotella) (Uyeno, Sekiguchi and Kamagata, 2008). Different research determined that 4 weeks of probiotic yoghurt consumption via healthy individuals improved intestinal content of probiotic *Lactobacilli* and *Bifidobacteria* strains, however, not significantly changing microbial population structure (Filteau *et al.*, 2013). Yoghurt consumption via children infected with Helicobacter pylori was determined to decrease the *H. pylori* load, restore an optimal *Bifidobacterium*⁄*E. coli* ratio and adjusted serum immune response (Yang and Sheu, 2012).

The use of antibiotics can eliminate the live microbes from joints, but it has to be given by injection. The oral administration of antibiotics can promote gut dysbiosis and worsen the arthritic state (Khan *et al.*, 2019; Wu *et al.*, 2020). Classical microbiological culture will be carried out to determine whether the human synovial fluid samples of patients with RA contained any viable bacterial cells; for instance, those capable of proliferation. Following bacterial growth in the media, single colonies from each plate will be selected for identification by total 16S gene amplification and then Sanger sequencing. After identification of type live bacteria, antibiotic sensitivity methods will be performed to determine which antibiotic will be most successful in treating a live bacterium in vivo.

As explained, a more invasive strategy that has produced promising outcomes is faecal microbiota transplantation (FMT), which directly alters microbial populations and their metabolites, and may directly or indirectly activate the host immune response.

It will be helpful to characterise the gut microbiome from the early stage, CIA mouse model by 16s rRNA, and ITS sequencing to identify the dysbiotic microbiome population in these subjects. Next, applying the faecal microbiota transplantation from healthy control subjects to normalise the dysbiotic intestinal microbiome population and examining whether this alteration changes the CIA disease process.

FMT is the transfer of an entire microbial population from a healthy donor to a diseased recipient in order to modify the recipient's microbial composition directly and confer a health benefit (Zhengxiao Zhang *et al.*, 2019). The first known description of the use of faeces as therapy was characterised via the 4th century by Ge Hong in China for the treatment of diseased conditions, including diarrhoea (Mcilroy *et al.*, 2019). In 1958, Eiseman and colleagues characterised the use of faecal enemas as a treatment for pseudomembranous colitis, considering the initiation of FMT into mainstream medicine (EISEMAN *et al.*, 1958). The process involves typically first picking a donor without a family history of metabolic, autoimmune, and malignant diseases and examine for any potential pathogens. The faeces materials are then made by mixing with normal saline or water, next, a filtration step to eliminate any particulate matter. The mixture can be applied through a nasojejunal tube, nasogastric tube esophagogastroduodenoscopy, retention enema, or colonoscopy. Most clinical practice with FMT has been derived from treating recurrent or refractory *Clostridium difficile* infection (RCDI) (Smits *et al.*, 2013). There is preliminary evidence to recommend that it may also offer the therapeutic potential for another disease, including inflammatory bowel disease (IBD), metabolic syndrome, and obesity (Szilagyi, 2020).

Recent investigations have revealed that FMT is an effective treatment for RCDI, with a higher than 90% success rate and can be regarded as an antibiotic replacement for RCDI (McBeth and Dobner, 2019). Due to the satisfactory outcome of FMT for RCDI, European Association of Infectious Diseases, European Association of Clinical Microbiology, and the American Society of Gastroenterology had entered FMT in the treatment guidelines of RCDI during 2013 and 2014, sequentially (Surawicz *et al.*, 2013; Debast *et al.*, 2014). This has encouraged studies on FMT as a possible therapy for other microbial- associated conditions such as IBD.

Since Bennet (Bennet and Brinkman, 1989) published the first case of FMT for the treatment of ulcerative colitis (UC) in 1989, lots of case reports, case series, and, more recently, randomised, controlled trials had been published regarding studying the efficiency and safety of FMT for IBD. Sun et al. (Sun *et al.* 2016) recorded that the percentage of clinical remission in UC patients was 30.4%. Moayyedi et al. (Moayyedi *et al.*, 2015) and Paramsothy et al. (Paramsothy *et al.*, 2017) revealed that the rate of clinical remission in patients who treated FMT was higher than that of placebo.

Shi et al. (Shi *et al.*, 2016) revealed that 41.58% of patients with UC achieved clinical remission, and 65.28% achieved a clinical response.

Further, a study published in JAMA in 2019 (Costello *et al.*, 2019), including 73 patients with mild to moderate UC, produced promising outcomes. The number of patients who experienced steroid-free remission at week eight was higher in the group which was treated with FMT rather than in the control group (32% vs 9%, P = 0.03). The overall response was also higher in the FMT treatment group rather than in the control group (55% vs 23%, P = 0.007).

A small double-blind, randomised, controlled research observed that faecal transplants from lean to obese (with metabolic syndrome) individuals produced in increased butyrate-producing bacteria (*Roseburia intestinalis*), improved insulin sensitivity, and enhanced gut-microbial diversity in the overweight recipients (Vrieze *et al.*, 2012).

While promising, the influence of FMT on long-term clinical endpoints needs to be explored. Further studies are also required to understand better the mechanisms through which changes in composition and function of the human microbiome affect disease processing for patients with inflammatory bowel disease, metabolic syndrome, and obesity (Zhengxiao Zhang *et al.*, 2019).

However, it is necessary to understand that FMT has a number of major limitations. For example, we do not know the best way of delivery (oral or rectal), or the frequency of FMT required producing durable responses. Furthermore, we have not distinguished specific advantageous taxa that can certainly attenuate different autoimmune conditions(Wilson *et al.*, 2019). Extensive donor testing is also needed to guarantee safety, as recent studies have shown transmission of drug-resistant organisms from donors to recipients (Borody, 2019; DeFilipp *et al.*, 2019).

7 Reference

Abdollahi-Roodsaz, S. *et al.* (2008) 'Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 118(1), pp. 205–16. doi: 10.1172/JCI32639.

Abdollahi-Roodsaz, S. *et al.* (2012) 'Destructive role of myeloid differentiation factor 88 and protective role of TRIF in interleukin-17-dependent arthritis in mice', *Arthritis & Rheumatism*, 64(6), pp. 1838–1847. doi: 10.1002/art.34328.

Abe, J. *et al.* (2015) 'Human Oral, Gut, and Blood Microbiota in Patients with Kawasaki Disease', *Am Heart Assoc*. Available at: https://www.ahajournals.org/doi/abs/10.1161/circ.131.suppl_2.39.

Abenavoli, L. *et al.* (2019) 'Gut microbiota and obesity: A role for probiotics', *Nutrients*. MDPI AG. doi: 10.3390/nu11112690.

Acharya, A. *et al.* (2017a) 'Salivary microbiome of an urban Indian cohort and patterns linked to subclinical inflammation', *Oral Diseases*, 23(7), pp. 926–940. doi: 10.1111/odi.12676.

Acharya, A. *et al.* (2017b) 'Salivary microbiome of an urban Indian cohort and patterns linked to subclinical inflammation', *Oral Diseases*, 23(7), pp. 926–940. doi: 10.1111/odi.12676.

Actor, J. K. and Actor, J. K. (2012) *Elsevier's integrated review. Immunology and microbiology*. Elsevier/Saunders.

Aggarwal, A., Sharma, A. and Bhatnagar, A. (2013) 'Bi(o)communications among peripheral blood fractions: A focus on NK and NKT cell biology in rheumatoid arthritis', *Autoimmunity*. doi: 10.3109/08916934.2012.755959.

Aggarwal, A., Sharma, A. and Bhatnagar, A. (2014) 'Role of cytolytic impairment of natural killer and natural killer T-cell populations in rheumatoid arthritis', *Clinical Rheumatology*. doi: 10.1007/s10067-014-2641-z.

Akbari, O. *et al.* (2003) 'Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity', *Nature Medicine*. doi: 10.1038/nm851.

Akkasheh, G. *et al.* (2016) 'Clinical and metabolic response to probiotic administration in patients with major depressive disorder: A randomized, double-blind, placebo-controlled trial', *Nutrition*. doi: 10.1016/j.nut.2015.09.003.

Al-Saadany, H. M. *et al.* (2016) 'Th-17 cells and serum IL-17 in rheumatoid arthritis patients: Correlation with disease activity and severity', *Egyptian Rheumatologist*. Egyptian Society for Joint Diseases and Arthritis, 38(1), pp. 1–7. doi: 10.1016/j.ejr.2015.01.001.

Alabarse, P. V. G. *et al.* (2018) 'Collagen-induced arthritis as an animal model of rheumatoid cachexia', *Journal of Cachexia, Sarcopenia and Muscle*. doi: 10.1002/jcsm.12280.

Alipour, B. *et al.* (2014) 'Effects of Lactobacillus casei supplementation on disease activity and inflammatory cytokines in rheumatoid arthritis patients: A randomized double-blind clinical trial', *International Journal of Rheumatic Diseases*. doi: 10.1111/1756-185X.12333.

Allender, M. C. *et al.* (2018) 'Snake fungal disease alters skin bacterial and fungal diversity in an endangered rattlesnake', *Scientific Reports*. Nature Publishing Group, 8(1), p. 12147. doi: 10.1038/s41598-018-30709-x.

Alunno, A. *et al.* (2015) 'Altered immunoregulation in rheumatoid arthritis: The role of regulatory T cells and proinflammatory Th17 cells and therapeutic implications', *Mediators of Inflammation*. Hindawi Limited. doi: 10.1155/2015/751793.

Amar, J. *et al.* (2011) 'Involvement of tissue bacteria in the onset of diabetes in humans: evidence for a concept', *Diabetologia*. Springer-Verlag, 54(12), pp. 3055–3061. doi: 10.1007/s00125-011-2329-8.

Amar, J. *et al.* (2013) 'Blood Microbiota Dysbiosis Is Associated with the Onset of Cardiovascular Events in a Large General Population: The D.E.S.I.R. Study', *PLoS ONE*. Edited by A. Bayer. Public Library of Science, 8(1), p. e54461. doi: 10.1371/journal.pone.0054461.

Ambers, A. *et al.* (2018) 'Direct PCR amplification of DNA from human bloodstains, saliva, and touch samples collected with microFLOQ® swabs', *Forensic Science International: Genetics*. doi: 10.1016/j.fsigen.2017.10.010.

Arleevskaya, M. I. *et al.* (2019) 'Toll-Like Receptors, Infections, and Rheumatoid Arthritis', *Clinical Reviews in Allergy and Immunology*. Humana Press Inc. doi: 10.1007/s12016-019-08742-z.

Arnett, F. C. *et al.* (1988) 'The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis', *Arthritis & Rheumatism*, 31(3), pp. 315–324. doi: 10.1002/art.1780310302.

Arrieta, M. C. *et al.* (2014) 'The intestinal microbiome in early life: Health and disease', *Frontiers in Immunology*. doi: 10.3389/fimmu.2014.00427.

Artis, D. (2008) 'Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut', *Nature Reviews Immunology*. Nature Publishing Group, 8(6), pp. 411–420. doi: 10.1038/nri2316.

Arumugam, M. *et al.* (2011) 'Enterotypes of the human gut microbiome', *Nature*. doi: 10.1038/nature09944.

Asaga, H., Yamada, M. and Senshu, T. (1998) 'Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages', *Biochemical and Biophysical Research Communications*. Academic Press Inc., 243(3), pp. 641–646. doi: 10.1006/bbrc.1998.8148.

Asquith, D. L. *et al.* (2009) 'Animal models of rheumatoid arthritis', *European Journal of Immunology*. John Wiley & Sons, Ltd, 39(8), pp. 2040–2044. doi: 10.1002/eji.200939578.

Asquith, M. *et al.* (2017) 'Intestinal Metabolites Are Profoundly Altered in the Context of HLA–B27 Expression and Functionally Modulate Disease in a Rat Model of Spondyloarthritis', *Arthritis and Rheumatology*. doi: 10.1002/art.40183.

Atarashi, K. *et al.* (2011) 'Induction of colonic regulatory T cells by indigenous Clostridium species.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 331(6015), pp. 337–41. doi: 10.1126/science.1198469.

Atarashi, K. *et al.* (2015) 'Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells', *Cell*, 163(2), pp. 367–380. doi: 10.1016/j.cell.2015.08.058.

Atianand, M. K. and Fitzgerald, K. A. (2013) 'Molecular Basis of DNA Recognition in the Immune System', *The Journal of Immunology*. doi: 10.4049/jimmunol.1203162.

Azevedo, V. F. and Buiar, P. G. (2013) 'Concurrent rheumatoid arthritis and ankylosing spondylitis in one patient: The importance of new classifi cation criteria', *Revista Brasileira de Reumatologia*. Elsevier Editora Ltda, 53(1), pp. 115–119. doi: 10.1590/S0482-50042013000100011.

Bach Knudsen, K. E. (2015) 'Microbial Degradation of Whole-Grain Complex Carbohydrates and Impact on Short-Chain Fatty Acids and Health', *Advances in Nutrition*. doi: 10.3945/an.114.007450.

Bäckhed, F. *et al.* (2005) 'Host-Bacterial Mutualism in the Human Intestine', *Science*. American Association for the Advancement of Science, 307(5717), pp. 1915–1920. doi: 10.1126/SCIENCE.1104816.

Bacon, C. M. *et al.* (1995) 'Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 92(16), pp. 7307–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7638186.

Baeuerle, P. A. and Henkel, T. (1994) 'Function and Activation of NF-kappaB in the Immune System', *Annual Review of Immunology*, 12(1), pp. 141–179. doi: 10.1146/annurev.iy.12.040194.001041.

Bakleh, M. *et al.* (2005) 'Infectious complications of tumor necrosis factor-α antagonists', *International Journal of Dermatology*. doi: 10.1111/j.1365-4632.2004.02571.x.

Baldwin, B. G. *et al.* (1995) 'The its Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny', *Annals of the Missouri Botanical Garden*. Missouri Botanical Garden Press, 82(2), p. 247. doi: 10.2307/2399880.

Barczyńska, T. A. *et al.* (2015) *Coexistence of rheumatoid arthritis and ankylosing spondylitis*, *Reumatologia*. doi: 10.5114/reum.2015.55832.

Barnes, P. J. and Karin, M. (1997) 'Nuclear Factor-κB — A Pivotal Transcription Factor in Chronic Inflammatory Diseases', *New England Journal of Medicine*. Edited by F. H. Epstein, 336(15), pp. 1066–1071. doi: 10.1056/NEJM199704103361506.

Bartold, P. M., Marshall, R. I. and Haynes, D. R. (2005) 'Periodontitis and Rheumatoid Arthritis: A Review', *Journal of Periodontology*, 76(11-s), pp. 2066–2074. doi: 10.1902/jop.2005.76.11-S.2066.

Bassis, C. M. *et al.* (2014) 'The nasal cavity microbiota of healthy adults', *Microbiome*. BioMed Central Ltd., 2(1). doi: 10.1186/2049-2618-2-27.

Baxter, A. G. *et al.* (1997) 'Association between alphabetaTCR+CD4-CD8- T-cell

deficiency and IDDM in NOD/Lt mice.', *Diabetes*. American Diabetes Association, 46(4), pp. 572–82. doi: 10.2337/DIAB.46.4.572.

Bazin, T. *et al.* (2018) 'Microbiota Composition May Predict Anti-TNF Alpha Response in Spondyloarthritis Patients: An Exploratory Study', *Scientific Reports*. doi: 10.1038/s41598-018-23571-4.

Beatty, M. *et al.* (2014) 'Small RNAs from plants, bacteria and fungi within the order Hypocreales are ubiquitous in human plasma', *BMC Genomics*. BioMed Central, 15(1), p. 933. doi: 10.1186/1471-2164-15-933.

Beck, J. M., Young, V. B. and Huffnagle, G. B. (2012) 'The Microbiome of the Lung', *Translational research : the journal of laboratory and clinical medicine*. NIH Public Access, 160(4), p. 258. doi: 10.1016/J.TRSL.2012.02.005.

Op De Beeck, M. *et al.* (2014) 'Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies.', *PloS one*. Public Library of Science, 9(6), p. e97629. doi: 10.1371/journal.pone.0097629.

Beirith, S. C., Ikino, C. M. Y. and Pereira, I. A. (2013) 'Laryngeal involvement in rheumatoid arthritis', *Brazilian Journal of Otorhinolaryngology*. Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico Facial, 79(2), pp. 233–238. doi: 10.5935/1808-8694.20130040.

Belkaid, Y. and Hand, T. W. (2014) 'Role of the microbiota in immunity and inflammation', *Cell*. doi: 10.1016/j.cell.2014.03.011.

Bendelac, A., Savage, P. B. and Teyton, L. (2007) 'The Biology of NKT Cells', *Annual Review of Immunology*. Annual Reviews, 25(1), pp. 297–336. doi: 10.1146/annurev.immunol.25.022106.141711.

Bennet, J. D. and Brinkman, M. (1989) 'TREATMENT OF ULCERATIVE COLITIS BY IMPLANTATION OF NORMAL COLONIC FLORA', *The Lancet*. doi: 10.1016/S0140-6736(89)91183-5.

BERGER, S. A. *et al.* (1974) 'Bacteremia After the Use of an Oral Irrigation Device', *Annals of Internal Medicine*. American College of Physicians, 80(4), p. 510. doi: 10.7326/0003-4819-80-4-510.

Beyer, K. *et al.* (2018) 'Subgingival microbiome of rheumatoid arthritis patients in relation to their disease status and periodontal health', *PLOS ONE*. Edited by M. Nurmohamed, 13(9), p. e0202278. doi: 10.1371/journal.pone.0202278.

Bibiloni, R. *et al.* (2005) 'Analysis of the large bowel microbiota of colitic mice using PCR/DGGE', *Letters in Applied Microbiology*. John Wiley & Sons, Ltd (10.1111), 41(1), pp. 45–51. doi: 10.1111/j.1472-765X.2005.01720.x.

Biedermann, L. *et al.* (2013) 'Smoking Cessation Induces Profound Changes in the Composition of the Intestinal Microbiota in Humans', *PLoS ONE*. doi: 10.1371/journal.pone.0059260.

Bjarnason, I. and Takeuchi, K. (2009) 'Intestinal permeability in the pathogenesis of NSAID-induced enteropathy', *Journal of Gastroenterology*. Springer Japan, 44(S19), pp. 23–29. doi: 10.1007/s00535-008-2266-6.

Blauvelt, A. and Chiricozzi, A. (2018) 'The Immunologic Role of IL-17 in Psoriasis and Psoriatic Arthritis Pathogenesis', *Clinical Reviews in Allergy & Immunology*, 55(3), pp. 379–390. doi: 10.1007/s12016-018-8702-3.

Blüml, S. *et al.* (2010) 'Antiinflammatory effects of tumor necrosis factor on hematopoietic cells in a murine model of erosive arthritis', *Arthritis and Rheumatism*. doi: 10.1002/art.27399.

Bodkhe, R., Balakrishnan, B. and Taneja, V. (2019a) 'The role of microbiome in rheumatoid arthritis treatment', *Therapeutic Advances in Musculoskeletal Disease*. SAGE Publications Ltd. doi: 10.1177/1759720X19844632.

Bodkhe, R., Balakrishnan, B. and Taneja, V. (2019b) 'The role of microbiome in rheumatoid arthritis treatment', *Therapeutic Advances in Musculoskeletal Disease*. SAGE Publications Ltd, p. 1759720X1984463. doi: 10.1177/1759720X19844632.

Boismenu, R. and Havran, W. L. (1994) 'Modulation of epithelial cell growth by intraepithelial γδT cells', *Science*. doi: 10.1126/science.7973709.

Boissier, M. C. *et al.* (2009) 'Regulatory T cells (Treg) in rheumatoid arthritis', *Joint Bone Spine*, pp. 10–14. doi: 10.1016/j.jbspin.2008.08.002.

Bokulich, N. A. and Mills, D. A. (2013) 'Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities', *Applied and Environmental Microbiology*, 79(8), pp. 2519–2526. doi: 10.1128/AEM.03870-12.

Bondeson, J. *et al.* (2006) 'The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis', *Arthritis Research and Therapy*, 8. doi: 10.1186/ar2099.

Bonilla, H. *et al.* (2011) 'Rapid diagnosis of septic arthritis using 16S rDNA PCR: a comparison of 3 methods', *Diagnostic Microbiology and Infectious Disease*. Elsevier, 69(4), pp. 390–395. doi: 10.1016/J.DIAGMICROBIO.2010.11.010.

Bonnet, E. *et al.* (2017) 'Joint infection due to Raoultella planticola: first report', *Infection*, 45(5), pp. 703–704. doi: 10.1007/s15010-017-1006-3.

Borger, P. *et al.* (1999) 'Proteases from *Aspergillus fumigatus* Induce Interleukin (IL)–6 and IL-8 Production in Airway Epithelial Cell Lines by Transcriptional Mechanisms', *The Journal of Infectious Diseases*, 180(4), pp. 1267–1274. doi: 10.1086/315027.

Borgo, F. *et al.* (2018) 'Body mass index and sex affect diverse microbial niches within the gut', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.00213.

Borody, T. (2019) 'Fecal microbiota transplantation', in *Gastrointestinal Diseases and Their Associated Infections*. doi: 10.1016/B978-0-323-54843-4.00018-0.

Le Bourhis, L. *et al.* (2010) 'Antimicrobial activity of mucosal-associated invariant T cells', *Nature Immunology*. doi: 10.1038/ni.1890.

Boyapati, A. *et al.* (2019) '069 High baseline serum IL-6 identifies a subgroup of rheumatoid arthritis patients with rapid joint damage and clinical progression and predicts

increased sarilumab treatment response', *Rheumatology*. Narnia, 58(Supplement_3). doi: 10.1093/rheumatology/kez106.068.

Brand, D. D., Latham, K. A. and Rosloniec, E. F. (2007a) 'Collagen-induced arthritis', *Nature Protocols*. Nature Publishing Group, 2(5), pp. 1269–1275. doi: 10.1038/nprot.2007.173.

Brand, D. D., Latham, K. A. and Rosloniec, E. F. (2007b) 'Collagen-induced arthritis', *Nature Protocols*. Nature Publishing Group, 2(5), pp. 1269–1275. doi: 10.1038/nprot.2007.173.

Brouqui, P. and Raoult, D. (2001) 'Endocarditis Due to Rare and Fastidious Bacteria', 14(1), pp. 177–207. doi: 10.1128/CMR.14.1.177-207.2001.

Brusca, S. B., Abramson, S. B. and Scher, J. U. (2014) 'Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity', *Current Opinion in Rheumatology*. doi: 10.1097/BOR.0000000000000008.

Bugatti, S. *et al.* (2014) 'B cells in rheumatoid arthritis: From pathogenic players to disease biomarkers', *BioMed Research International*. Hindawi Publishing Corporation, 2014. doi: 10.1155/2014/681678.

Bunker, J. J. *et al.* (2015) 'Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A', *Immunity*. doi: 10.1016/j.immuni.2015.08.007.

Buonocore, S. *et al.* (2010) 'Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology', *Nature*. doi: 10.1038/nature08949.

Burd, E. M. and Westblade, L. F. (2017) 'The Bacterial Blood Microbiota/Microbiome', in *The Dark Art of Blood Cultures*. American Society of Microbiology, pp. 277–295. doi: 10.1128/9781555819811.ch13.

Burska, A., Boissinot, M. and Ponchel, F. (2014) 'Cytokines as biomarkers in rheumatoid arthritis.', *Mediators of inflammation*. Hindawi Limited, 2014, p. 545493. doi: 10.1155/2014/545493.

Bustamante, M. F. *et al.* (2020) 'Design of an anti-inflammatory diet (ITIS diet) for patients with rheumatoid arthritis', *Contemporary Clinical Trials Communications*. doi: 10.1016/j.conctc.2020.100524.

Byrd, A. L., Belkaid, Y. and Segre, J. A. (2018) 'The human skin microbiome', *Nature Reviews Microbiology*. Nature Publishing Group, pp. 143–155. doi: 10.1038/nrmicro.2017.157.

Cai, L. *et al.* (2001) 'Pathways by which interleukin 17 induces articular cartilage breakdown in vitro and in vivo', *Cytokine*. doi: 10.1006/cyto.2001.0939.

Campos, F. P. F. de, Guimarães, T. B. and Lovisolo, S. M. (2016) 'Fatal pancreatic pseudocyst co-infected by Raoultella planticola: an emerging pathogen', *Autopsy and Case Reports*, 6(2), pp. 27–31. doi: 10.4322/acr.2016.034.

Canavan, M. *et al.* (2015) 'A1.14 Characterisation and activation of dendritic cell subsets within the inflammed synovium of rheumatoid arthritis patients', *Annals of the Rheumatic*

Diseases. doi: 10.1136/annrheumdis-2015-207259.14.

Caporaso, J. G. *et al.* (2010) 'QIIME allows analysis of high-throughput community sequencing data', *Nature Methods*. Nature Publishing Group, 7(5), pp. 335–336. doi: 10.1038/nmeth.f.303.

Carr, M. J. *et al.* (2005) 'First reported case of endocarditis caused by Candida dubliniensis.', *Journal of clinical microbiology*. American Society for Microbiology Journals, 43(6), pp. 3023–6. doi: 10.1128/JCM.43.6.3023-3026.2005.

Castañeda-Delgado, J. E. *et al.* (2017) 'Type I interferon gene response is increased in early and established rheumatoid arthritis and correlates with autoantibody production', *Frontiers in Immunology*. doi: 10.3389/fimmu.2017.00285.

Castillo, D. J. *et al.* (2019) 'The Healthy Human Blood Microbiome: Fact or Fiction?', *Frontiers in cellular and infection microbiology*. Frontiers Media SA, 9, p. 148. doi: 10.3389/fcimb.2019.00148.

Catrina, A. I. *et al.* (2014) 'Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis', *Nature Reviews Rheumatology*. Nature Publishing Group, 10(11), pp. 645–653. doi: 10.1038/nrrheum.2014.115.

Cella, M. *et al.* (2009) 'A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity', *Nature*. doi: 10.1038/nature07537.

Chabaud, M. *et al.* (1998) 'Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines.', *Journal of immunology (Baltimore, Md. : 1950)*. doi: 10.4049/jimmunol.165.9.5332.

Chabot, S. *et al.* (2006) 'TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 176(7), pp. 4275–83. doi: 10.4049/JIMMUNOL.176.7.4275.

Chen, J. *et al.* (2016) 'An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis', *Genome Medicine*, 8(1), p. 43. doi: 10.1186/s13073-016-0299-7.

Chen, V. L. and Kasper, D. L. (2014) 'Interactions between the intestinal microbiota and innate lymphoid cells', *Gut Microbes*. Taylor & Francis, 5(1), pp. 129–140. doi: 10.4161/gmic.27289.

Chen, W.-S. *et al.* (2012) 'Association of serum interleukin-17 and interleukin-23 levels with disease activity in Chinese patients with ankylosing spondylitis', *Journal of the Chinese Medical Association*. No longer published by Elsevier, 75(7), pp. 303–308. doi: 10.1016/J.JCMA.2012.05.006.

Cheng, Z., Meade, J. and Devine, D. A. (2017) 'Periodontal disease and periodontal bacteria as triggers for rheumatoid arthritis', *Best Practice & Research Clinical Rheumatology*. Baillière Tindall, 31(1), pp. 19–30. doi: 10.1016/J.BERH.2017.08.001.

Cheung, S. G. *et al.* (2019) 'Systematic review of gut microbiota and major depression', *Frontiers in Psychiatry*. Frontiers Media S.A. doi: 10.3389/fpsyt.2019.00034.

Chi, H. and Flavell, R. A. (2008) 'Innate recognition of non-self nucleic acids', *Genome*

Biology. doi: 10.1186/gb-2008-9-3-211.

Cho, Y.-N. *et al.* (2014) 'Mucosal-Associated Invariant T Cell Deficiency in Systemic Lupus Erythematosus', *The Journal of Immunology*. doi: 10.4049/jimmunol.1302701.

Choi, I. A. *et al.* (2016) 'Periodontitis is associated with rheumatoid arthritis: a study with longstanding rheumatoid arthritis patients in Korea.', *The Korean journal of internal medicine*. Korean Association of Internal Medicine, 31(5), pp. 977–86. doi: 10.3904/kjim.2015.202.

Choi, J. H. *et al.* (2017) 'IgD class switching is initiated by microbiota and limited to mucosa-associated lymphoid tissue in mice', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1621258114.

Choi, S.-T. and Lee, K.-H. (2018) 'Clinical management of seronegative and seropositive rheumatoid arthritis: A comparative study', *PLOS ONE*. Edited by T. Kato, 13(4), p. e0195550. doi: 10.1371/journal.pone.0195550.

Choudhary, N., Bhatt, L. K. and Prabhavalkar, K. S. (2018) 'Experimental animal models for rheumatoid arthritis', *Immunopharmacology and Immunotoxicology*, 40(3), pp. 193–200. doi: 10.1080/08923973.2018.1434793.

Choy, E. (2012) 'Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis', *Rheumatology*. Oxford University Press, 51(suppl 5), pp. v3–v11. doi: 10.1093/rheumatology/kes113.

Christensen, L. *et al.* (2019) 'Prevotella Abundance Predicts Weight Loss Success in Healthy, Overweight Adults Consuming a Whole-Grain Diet Ad Libitum: A Post Hoc Analysis of a 6-Wk Randomized Controlled Trial', *Journal of Nutrition*. doi: 10.1093/jn/nxz198.

Chu, D. M. *et al.* (2017) 'Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery', *Nature Medicine*. Nature Publishing Group, 23(3), pp. 314–326. doi: 10.1038/nm.4272.

Clarke, S. F. *et al.* (2012) 'The gut microbiota and its relationship to diet and obesity:New insights', *Gut Microbes*. Landes Bioscience, pp. 186–202. doi: 10.4161/gmic.20168.

Clarridge, J. E. (2004) 'Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases.', *Clinical microbiology reviews*. American Society for Microbiology Journals, 17(4), pp. 840–62, table of contents. doi: 10.1128/CMR.17.4.840-862.2004.

Cloutier, J. F. and Veillette, A. (1999) 'Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase.', *The Journal of experimental medicine*. The Rockefeller University Press, 189(1), pp. 111–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9874568.

Coenen, D. *et al.* (2007) 'Technical and Diagnostic Performance of 6 Assays for the Measurement of Citrullinated Protein/Peptide Antibodies in the Diagnosis of Rheumatoid Arthritis', *Clinical Chemistry*, 53(3), pp. 498–504. doi: 10.1373/clinchem.2006.078063.

Cohen, C. *et al.* (2015) 'Similarities and seasonal variations in bacterial communities from the blood of rodents and from their flea vectors', *The ISME Journal*, 9(7), pp. 1662–1676.

doi: 10.1038/ismej.2014.255.

Collins, M. D. *et al.* (1999) 'Characterization of a novel Gram-positive, catalase-negative coccus from horses: description of Eremococcus coleocola gen. nov. sp. nov.', *International Journal of Systematic Bacteriology*, 49(4), pp. 1381–1385. doi: 10.1099/00207713-49-4-1381.

Cooles, F. A. H., Isaacs, J. D. and Anderson, A. E. (2013) 'Treg cells in rheumatoid arthritis: An update', *Current Rheumatology Reports*, 15(9). doi: 10.1007/s11926-013-0352-0.

Cooper, D. *et al.* (2016) 'Relationship between human gut microbiota and interleukin 6 levels in overweight and obese adults', *fasebj.org*. Available at: https://www.fasebj.org/doi/abs/10.1096/fasebj.30.1_supplement.146.4.

Cope, A. P. (2008) 'T cells in rheumatoid arthritis', *Arthritis Research and Therapy*. doi: 10.1186/ar2412.

Coppieters, K. *et al.* (2007) 'A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner.', *Journal of Immunology*.

Costello, M.-E. *et al.* (2013) 'Microbes, the gut and ankylosing spondylitis', *Arthritis Research & Therapy*. BioMed Central, 15(3), p. 214. doi: 10.1186/ar4228.

Costello, S. P. *et al.* (2019) 'Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients with Ulcerative Colitis: A Randomized Clinical Trial', in *JAMA - Journal of the American Medical Association*. doi: 10.1001/jama.2018.20046.

Cruz, C. M. *et al.* (2007) 'ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 282(5), pp. 2871–9. doi: 10.1074/jbc.M608083200.

Cua, D. J. and Tato, C. M. (2010) 'Innate IL-17-producing cells: The sentinels of the immune system', *Nature Reviews Immunology*. doi: 10.1038/nri2800.

Cunnington, A. J. *et al.* (2016) '"Vaginal seeding" of infants born by caesarean section', *BMJ*. doi: 10.1136/bmj.i227.

D'Alessandro-Gabazza, C. N. *et al.* (2018) 'Identification of Halophilic Microbes in Lung Fibrotic Tissue by Oligotyping', *Frontiers in Microbiology*. Frontiers, 9, p. 1892. doi: 10.3389/fmicb.2018.01892.

D'Argenio, V. and Salvatore, F. (2015) 'The role of the gut microbiome in the healthy adult status', *Clinica Chimica Acta*, 451, pp. 97–102. doi: 10.1016/j.cca.2015.01.003.

Daliri, E. B.-M. and Lee, B. H. (2015) 'New perspectives on probiotics in health and disease', *Food Science and Human Wellness*. doi: 10.1016/j.fshw.2015.06.002.

Damgaard, C. *et al.* (2015) 'Viable Bacteria Associated with Red Blood Cells and Plasma in Freshly Drawn Blood Donations', *PLOS ONE*. Edited by L. Gregori. Public Library of Science, 10(3), p. e0120826. doi: 10.1371/journal.pone.0120826.

Danska, J. S. (2014a) 'Sex matters for mechanism', *Science Translational Medicine*. American Association for the Advancement of Science, p. 258fs40. doi: 10.1126/scitranslmed.3009859.

Danska, J. S. (2014b) 'Sex matters for mechanism', *Science Translational Medicine*. doi: 10.1126/scitranslmed.3009859.

Debast, S. B. *et al.* (2014) 'European society of clinical microbiology and infectious diseases: Update of the treatment guidance document for Clostridium difficile infection', *Clinical Microbiology and Infection*. doi: 10.1111/1469-0691.12418.

DeFilipp, Z. *et al.* (2019) 'Drug-resistant e. coli bacteremia transmitted by fecal microbiota transplant', *New England Journal of Medicine*. doi: 10.1056/NEJMoa1910437.

Depcik-Smith, N. D., Hay, S. N. and Brecher, M. E. (2001) 'Bacterial contamination of blood products: Factors, options, and insights', *Journal of Clinical Apheresis*. John Wiley & Sons, Ltd, 16(4), pp. 192–201. doi: 10.1002/jca.10004.

Derksen, V. F. A. M., Huizinga, T. W. J. and van der Woude, D. (2017) 'The role of autoantibodies in the pathophysiology of rheumatoid arthritis', *Seminars in Immunopathology*. Springer Verlag, pp. 437–446. doi: 10.1007/s00281-017-0627-z.

Derrien, M. and Veiga, P. (2017) 'Rethinking Diet to Aid Human–Microbe Symbiosis', *Trends in Microbiology*. doi: 10.1016/j.tim.2016.09.011.

Dethlefsen, L. and Relman, D. A. (2011) 'Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1000087107.

Dhaenens, M. *et al.* (2009) 'Dendritic cells from spondylarthritis-prone HLA-B27-transgenic rats display altered cytoskeletal dynamics, class II major histocompatibility complex expression, and viability', *Arthritis and Rheumatism*, 60(9), pp. 2622–2632. doi: 10.1002/art.24780.

Diamanti, A. P. *et al.* (2016) 'Microbiota and chronic inflammatory arthritis: An interwoven link', *Journal of Translational Medicine*. doi: 10.1186/s12967-016-0989-3.

Dinarello, C. A., Simon, A. and Van Der Meer, J. W. M. (2012) 'Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases', *Nature Reviews Drug Discovery*. doi: 10.1038/nrd3800.

Ding, R. xue *et al.* (2019) 'Revisit gut microbiota and its impact on human health and disease', *Journal of Food and Drug Analysis*. Elsevier Taiwan LLC, pp. 623–631. doi: 10.1016/j.jfda.2018.12.012.

Dixit, A., Bhardwaj, M. and Sharma, B. (2012) 'Headache in Pregnancy: A Nuisance or a New Sense?', *Obstetrics and Gynecology International*. Hindawi, 2012, pp. 1–11. doi: 10.1155/2012/697697.

Dominguez-Bello, M. G. *et al.* (2010) 'Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns', *Proceedings of the National Academy of Sciences of the United States of America*, 107(26), pp. 11971–11975. doi: 10.1073/pnas.1002601107.

Dominianni, C. *et al.* (2015) 'Sex, body mass index, and dietary fiber intake influence the human gut microbiome', *PLoS ONE*. doi: 10.1371/journal.pone.0124599.

Douzandeh-Mobarrez, B. and Kariminik, A. (2017) 'Gut Microbiota and IL-17A: Physiological and Pathological Responses', *Probiotics and Antimicrobial Proteins*. doi: 10.1007/s12602-017-9329-z.

Drago, L. *et al.* (2019) 'Oral–Gut Microbiota and Arthritis: Is There an Evidence-Based Axis?', *Journal of Clinical Medicine*, 8(10), p. 1753. doi: 10.3390/jcm8101753.

Duan, J. *et al.* (2010) 'Microbial Colonization Drives Expansion of IL-1 Receptor 1-Expressing and IL-17-Producing γ/δ T Cells', *Cell Host & Microbe*. Cell Press, 7(2), pp. 140–150. doi: 10.1016/J.CHOM.2010.01.005.

Dupuy, A. K. *et al.* (2014) 'Redefining the Human Oral Mycobiome with Improved Practices in Amplicon-based Taxonomy: Discovery of Malassezia as a Prominent Commensal', *PLoS ONE*. Edited by D. R. Andes. Public Library of Science, 9(3), p. e90899. doi: 10.1371/journal.pone.0090899.

Dusseaux, M. *et al.* (2011) 'Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161 hi IL-17-secreting T cells', *Blood*. doi: 10.1182/blood-2010-08-303339.

Ebringer, A. and Rashid, T. (2006) 'Rheumatoid arthritis is an autoimmune disease triggered by Proteus urinary tract infection.', *Clinical & developmental immunology*. Hindawi, 13(1), pp. 41–8. doi: 10.1080/17402520600576578.

Eerola, E. *et al.* (1994) 'Intestinal flora in early rheumatoid arthritis.', *British journal of rheumatology*, 33(11), pp. 1030–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7981990.

Ehrenstein, M. R. and Notley, C. A. (2010) 'The importance of natural IgM: Scavenger, protector and regulator', *Nature Reviews Immunology*. doi: 10.1038/nri2849.

EISEMAN, B. *et al.* (1958) 'Fecal enema as an adjunct in the treatment of pseudomembranous', *Surgery*.

Eisenhofer, R. *et al.* (2019) 'Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations', *Trends in Microbiology*. Elsevier Current Trends, 27(2), pp. 105–117. doi: 10.1016/J.TIM.2018.11.003.

Elderman, M., de Vos, P. and Faas, M. (2018) 'Role of microbiota in sexually dimorphic immunity', *Frontiers in Immunology*. doi: 10.3389/fimmu.2018.01018.

Elhewala, A. E. *et al.* (2015) 'Interleukin-17 level in rheumatoid arthritis patients and its relation to disease activity: a clinical and ultrasound study', *Egyptian Rheumatology and Rehabilitation*. Medknow Publications and Media Pvt. Ltd., 42(4), p. 183. doi: 10.4103/1110-161X.168164.

Elkayam, O. *et al.* (2000) 'Serum levels of IL-10, IL-6, IL-1ra, and sIL-2R in patients with psoriatic arthritis', *Rheumatology International*. Springer-Verlag, 19(3), pp. 101–105. doi: 10.1007/s002960050111.

Erb-Downward, J. R. *et al.* (2011) 'Analysis of the Lung Microbiome in the "Healthy" Smoker and in COPD', *PLoS ONE*. Edited by S. Bereswill. Public Library of Science, 6(2),

p. e16384. doi: 10.1371/journal.pone.0016384.

Eriksson, J. E. *et al.* (2009) 'Introducing intermediate filaments: From discovery to disease', *Journal of Clinical Investigation*. The American Society for Clinical Investigation, pp. 1763–1771. doi: 10.1172/JCI38339.

Eto, D. *et al.* (2011) 'IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation', *PLoS ONE*. Edited by L. N. F. Poh. Public Library of Science, 6(3), p. e17739. doi: 10.1371/journal.pone.0017739.

Evans, D. M. *et al.* (2011) 'Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility', *Nature Genetics*. doi: 10.1038/ng.873.

Ferrer Bradley, I. *et al.* (2018) 'Pulmonary aspergillosis in a Crohn's disease patient receiving adalimumab and steroid therapy.', *Gastroenterología y Hepatología*. doi: 10.1016/j.gastrohep.2018.05.028.

Fert, I. *et al.* (2008) 'Correlation between dendritic cell functional defect and spondylarthritis phenotypes in HLA-B27/human β2-microglobulin-transgenic rat lines', *Arthritis and Rheumatism*. John Wiley & Sons, Ltd, 58(11), pp. 3425–3429. doi: 10.1002/art.24023.

De Filippo, C. *et al.* (2010) 'Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1005963107.

Filteau, M. *et al.* (2013) 'Molecular monitoring of fecal microbiota in healthy adults following probiotic yogurt intake', *PharmaNutrition*. doi: 10.1016/j.phanu.2013.05.002.

Fine, L. *et al.* (2019) 'Investigation of a Serratia Marcescens bacteremia Cluster in a Cardiothoracic Intensive Care Unit (CICU) Utilizing Whole Genome Sequencing', *American Journal of Infection Control*. Mosby, 47(6), pp. S10–S11. doi: 10.1016/J.AJIC.2019.04.157.

Forner, L. *et al.* (2006) 'Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation', *Journal of Clinical Periodontology*. John Wiley & Sons, Ltd (10.1111), 33(6), pp. 401–407. doi: 10.1111/j.1600-051X.2006.00924.x.

Frank, J. *et al.* (2003) 'Transforming Growth Factor-β1 Decreases Expression of the Epithelial Sodium Channel αENaC and Alveolar Epithelial Vectorial Sodium and Fluid Transport via an ERK1/2-dependent Mechanism', *Journal of Biological Chemistry*, 278(45), pp. 43939–43950. doi: 10.1074/jbc.M304882200.

Fransen, F. *et al.* (2015) 'BALB/c and C57BL/6 Mice Differ in Polyreactive IgA Abundance, which Impacts the Generation of Antigen-Specific IgA and Microbiota Diversity', *Immunity*. doi: 10.1016/j.immuni.2015.08.011.

Fransen, F. *et al.* (2017) 'The impact of gut microbiota on gender-specific differences in immunity', *Frontiers in Immunology*. doi: 10.3389/fimmu.2017.00754.

Fujiwara, D. *et al.* (2008) 'Systemic control of plasmacytoid dendritic cells by CD8+ T cells and commensal microbiota.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 180(9), pp. 5843–52. doi: 10.4049/JIMMUNOL.180.9.5843.

Fulbright, L. E., Ellermann, M. and Arthur, J. C. (2017) 'The microbiome and the hallmarks of cancer', *PLOS Pathogens*. Edited by J. M. Leong. Public Library of Science, 13(9), p. e1006480. doi: 10.1371/journal.ppat.1006480.

Fung, T. C. *et al.* (2016a) 'Lymphoid-Tissue-Resident Commensal Bacteria Promote Members of the IL-10 Cytokine Family to Establish Mutualism', *Immunity*, 44(3), pp. 634–646. doi: 10.1016/j.immuni.2016.02.019.

Fung, T. C. *et al.* (2016b) 'Lymphoid-Tissue-Resident Commensal Bacteria Promote Members of the IL-10 Cytokine Family to Establish Mutualism', *Immunity*, 44(3), pp. 634–646. doi: 10.1016/j.immuni.2016.02.019.

Fung, T. C., Artis, D. and Sonnenberg, G. F. (2014) 'Anatomical localization of commensal bacteria in immune cell homeostasis and disease', *Immunological Reviews*. doi: 10.1111/imr.12186.

Gaboriau-Routhiau, V. *et al.* (2009) 'The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses', *Immunity*. Cell Press, 31(4), pp. 677–689. doi: 10.1016/J.IMMUNI.2009.08.020.

Gabriel, S. E. (2008) 'Why do people with rheumatoid arthritis still die prematurely?', *Annals of the Rheumatic Diseases*, 67(Suppl 3), pp. iii30–iii34. doi: 10.1136/ard.2008.098038.

Gade, L. *et al.* (2013) 'Detection of fungal DNA in human body fluids and tissues during a multistate outbreak of fungal meningitis and other infections.', *Eukaryotic cell*. American Society for Microbiology (ASM), 12(5), pp. 677–83. doi: 10.1128/EC.00046-13.

Gaffen, S. L. (2009a) 'Role of IL-17 in the Pathogenesis of Rheumatoid Arthritis', *Current rheumatology reports*. NIH Public Access, 11(5), p. 365. doi: 10.1007/S11926-009-0052-Y.

Gaffen, S. L. (2009b) 'The role of interleukin-17 in the pathogenesis of rheumatoid arthritis', *Current Rheumatology Reports*, pp. 365–370. doi: 10.1007/s11926-009-0052-y.

Gajdács, M. *et al.* (2019) 'Resistance Trends and Epidemiology of Citrobacter-Enterobacter-Serratia in Urinary Tract Infections of Inpatients and Outpatients (RECESUTI): A 10-Year Survey', *Medicina*. Multidisciplinary Digital Publishing Institute, 55(6), p. 285. doi: 10.3390/medicina55060285.

Gaur, P., Misra, R. and Aggarwal, A. (2015) 'Natural killer cell and gamma delta T cell alterations in enthesitis related arthritis category of juvenile idiopathic arthritis', *Clinical Immunology*. doi: 10.1016/j.clim.2015.07.012.

Ge, X. *et al.* (2017) 'Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility', *Journal of Translational Medicine*. doi: 10.1186/s12967-016-1105-4.

Genco, R. J. *et al.* (2005) 'A Proposed Model Linking Inflammation to Obesity, Diabetes, and Periodontal Infections', *Journal of Periodontology*, 76(11-s), pp. 2075–2084. doi: 10.1902/jop.2005.76.11-S.2075.

Gevers, D. *et al.* (2012) 'The Human Microbiome Project: A Community Resource for the Healthy Human Microbiome', *PLoS Biology*. Public Library of Science, 10(8), p. e1001377. doi: 10.1371/journal.pbio.1001377.

Ghannoum, M. A. *et al.* (2010) 'Characterization of the Oral Fungal Microbiome (Mycobiome) in Healthy Individuals', *PLoS Pathogens*. Edited by R. C. May. Public Library of Science, 6(1), p. e1000713. doi: 10.1371/journal.ppat.1000713.

Ghosh, T. S. *et al.* (2020) 'Adjusting for age improves identification of gut microbiome alterations in multiple diseases', *eLife*. eLife Sciences Publications Ltd, 9. doi: 10.7554/eLife.50240.

Gierut, A., Perlman, H. and North, R. P.-R. D. C. (2010) 'Innate Immunity and Rheumatoid Arthritis', *Elsevier*, pp. 271–296.

Giles, J. T. and Bathon, J. M. (2004) 'Serious infections associated with anticytokine therapies in the rheumatic diseases', *Journal of Intensive Care Medicine*. doi: 10.1177/0885066604267854.

Gilis, E. *et al.* (2018) 'The Role of the Microbiome in Gut and Joint Inflammation in Psoriatic Arthritis and Spondyloarthritis.', *The Journal of rheumatology. Supplement*. The Journal of Rheumatology, 94, pp. 36–39. doi: 10.3899/jrheum.180135.

Girbal-Neuhauser, E. *et al.* (1999) 'The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues.', *Journal of immunology (Baltimore, Md. : 1950)*.

Gladman, D. D. (2015) 'Clinical Features and Diagnostic Considerations in Psoriatic Arthritis', *Rheumatic Disease Clinics of North America*. doi: 10.1016/j.rdc.2015.07.003.

Glassing, A. *et al.* (2016) 'Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples', *Gut Pathogens*. BioMed Central, 8(1), p. 24. doi: 10.1186/s13099-016-0103-7.

Gloor, G. B. *et al.* (2018) 'Data on the gut and saliva microbiota from a cohort of atherosclerosis patients determined by 16S rRNA gene sequencing.', *Data in brief*. Elsevier, 19, pp. 481–485. doi: 10.1016/j.dib.2018.05.032.

Gold, M. C. *et al.* (2010) 'Human mucosal associated invariant T cells detect bacterially infected cells', *PLoS Biology*. doi: 10.1371/journal.pbio.1000407.

Gomez, A. *et al.* (2012) 'Loss of sex and age driven differences in the gut microbiome characterize arthritis-susceptible *0401 mice but not arthritis-resistant *0402 mice', *PLoS ONE*. doi: 10.1371/journal.pone.0036095.

Goodrich, J. K. *et al.* (2014) 'Conducting a microbiome study', *Cell*. Cell Press, pp. 250–262. doi: 10.1016/j.cell.2014.06.037.

Gosiewski, T. *et al.* (2017) 'Comprehensive detection and identification of bacterial DNA in the blood of patients with sepsis and healthy volunteers using next-generation sequencing method - the observation of DNAemia', *European Journal of Clinical Microbiology & Infectious Diseases*. Springer Berlin Heidelberg, 36(2), pp. 329–336. doi: 10.1007/s10096-016-2805-7.

Granfors, K. *et al.* (1998) 'Persistence ofYersinia antigens in peripheral blood cells from patients withYersinia enterocolitica O:3 infection with or without reactive arthritis', *Arthritis & Rheumatism*. John Wiley & Sons, Ltd, 41(5), pp. 855–862. doi: 10.1002/1529-

0131(199805)41:5<855::AID-ART12>3.0.CO;2-J.

Grembi, J. A. *et al.* (2020) 'Gut microbiota plasticity is correlated with sustained weight loss on a low-carb or low-fat dietary intervention', *Scientific Reports*. doi: 10.1038/s41598-020-58000-y.

Grice, Elizabeth A. *et al.* (2008) 'A diversity profile of the human skin microbiota', *Genome Research*. doi: 10.1101/gr.075549.107.

Grice, E. A. *et al.* (2008) 'A diversity profile of the human skin microbiota', *Genome Research*, 18(7), pp. 1043–1050. doi: 10.1101/gr.075549.107.

Grice, E. A. *et al.* (2009) 'Topographical and temporal diversity of the human skin microbiome', *Science*. American Association for the Advancement of Science, 324(5931), pp. 1190–1192. doi: 10.1126/science.1171700.

Grice, E. A. and Segre, J. A. (2012) 'The Human Microbiome: Our Second Genome', *Annual Review of Genomics and Human Genetics*. Annual Reviews , 13(1), pp. 151–170. doi: 10.1146/annurev-genom-090711-163814.

Guerne, P. A. *et al.* (1989) 'Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 83(2), pp. 585–92. doi: 10.1172/JCI113921.

Gupta, V. *et al.* (2017) 'Immunology and Medical Microbiology', in *Basic and Applied Aspects of Biotechnology*. doi: 10.1007/978-981-10-0875-7_8.

Gupta, V. K., Paul, S. and Dutta, C. (2017) 'Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.01162.

G�rard, H. C. *et al.* (2001) 'Chromosomal DNA from a variety of bacterial species is present in synovial tissue from patients with various forms of arthritis', *Arthritis & Rheumatism*. John Wiley & Sons, Ltd, 44(7), pp. 1689–1697. doi: 10.1002/1529-0131(200107)44:7<1689::AID-ART293>3.0.CO;2-K.

Hall, M. N. (2008) 'mTOR—What Does It Do?', *Transplantation Proceedings*, 40(10), pp. S5–S8. doi: 10.1016/j.transproceed.2008.10.009.

Hallen-Adams, H. E. and Suhr, M. J. (2016) 'Fungi in the healthy human gastrointestinal tract'. doi: 10.1080/21505594.2016.1247140.

Hallen-Adams, H. E. and Suhr, M. J. (2017) 'Fungi in the healthy human gastrointestinal tract', *Virulence*, 8(3), pp. 352–358. doi: 10.1080/21505594.2016.1247140.

Haller, D. (ed.) (2018) *The Gut Microbiome in Health and Disease*. Cham: Springer International Publishing. doi: 10.1007/978-3-319-90545-7.

Hamaguchi, K. *et al.* (2012) 'Analysis of adipose tissues and stromal vascular cells in a murine arthritis model', *Metabolism: Clinical and Experimental*. doi: 10.1016/j.metabol.2012.05.018.

Hammad, D. B. M. *et al.* (2020) 'Molecular Characterization of Circulating Microbiome Signatures in Rheumatoid Arthritis', *Frontiers in Cellular and Infection Microbiology*. doi:

10.3389/fcimb.2019.00440.

Hammad, D. B. M., Liyanapathirana, V. and Tonge, D. P. (2019) 'Molecular characterisation of the synovial fluid microbiome in rheumatoid arthritis patients and healthy control subjects', *PLoS ONE*. doi: 10.1371/journal.pone.0225110.

Haro, C. *et al.* (2016) 'Intestinal Microbiota Is Influenced by Gender and Body Mass Index', *PLOS ONE*. Edited by Y. Sanz. Public Library of Science, 11(5), p. e0154090. doi: 10.1371/journal.pone.0154090.

Harris, K. A. *et al.* (2002) 'Unique case of Helicobacter sp. osteomyelitis in an immunocompetent child diagnosed by broad-range 16S PCR.', *Journal of clinical microbiology*. American Society for Microbiology Journals, 40(8), pp. 3100–3. doi: 10.1128/JCM.40.8.3100-3103.2002.

Hartley, J. C. and Harris, K. A. (2003) 'Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service', *Journal of Medical Microbiology*, 52(8), pp. 685–691. doi: 10.1099/jmm.0.05213-0.

Hasan, N. and Yang, H. (2019) 'Factors affecting the composition of the gut microbiota, and its modulation', *PeerJ*. PeerJ Inc. doi: 10.7717/peerj.7502.

Hasegawa, M. *et al.* (2006) 'Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M602638200.

Hatakka, K. *et al.* (2003) 'Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis - A pilot study', *Scandinavian Journal of Rheumatology*. doi: 10.1080/03009740310003695.

Helgeland, L. *et al.* (2004) 'Microbial colonization induces oligoclonal expansions of intraepithelial CD8 T cells in the gut', *European Journal of Immunology*. John Wiley & Sons, Ltd, 34(12), pp. 3389–3400. doi: 10.1002/eji.200425122.

Heller, F. *et al.* (2002) 'Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells', *Immunity*. doi: 10.1016/S1074-7613(02)00453-3.

Henning, S. M. *et al.* (2018) 'Decaffeinated green and black tea polyphenols decrease weight gain and alter microbiome populations and function in diet-induced obese mice', *European Journal of Nutrition*. Dr. Dietrich Steinkopff Verlag GmbH and Co. KG, 57(8), pp. 2759–2769. doi: 10.1007/s00394-017-1542-8.

Hernandez, C. J. (2017) 'The Microbiome and Bone and Joint Disease', *Current Rheumatology Reports*. Current Medicine Group LLC 1, pp. 1–6. doi: 10.1007/s11926-017-0705-1.

Hilty, M. *et al.* (2010) 'Disordered Microbial Communities in Asthmatic Airways', *PLoS ONE*. Public Library of Science, 5(1). doi: 10.1371/JOURNAL.PONE.0008578.

Hippe, B. *et al.* (2015) 'SCFA Producing Gut Microbiota and its Effects on the Epigenetic Regulation of Inflammation', in, pp. 181–197. doi: 10.1007/978-3-319-23213-3_9.

Hirano, T. *et al.* (1988) 'Excessive production of interleukin 6/B cell stimulatory factor-2 in

rheumatoid arthritis', *European Journal of Immunology*. John Wiley & Sons, Ltd, 18(11), pp. 1797–1802. doi: 10.1002/eji.1830181122.

Hitchon, C. A. *et al.* (2010) 'Antibodies to porphyromonas gingivalis are associated with anticitrullinated protein antibodies in patients with rheumatoid arthritis and their relatives.', *The Journal of rheumatology*. The Journal of Rheumatology, 37(6), pp. 1105–12. doi: 10.3899/jrheum.091323.

Hoggard, M. *et al.* (2018) 'Characterizing the Human Mycobiota: A Comparison of Small Subunit rRNA, ITS1, ITS2, and Large Subunit rRNA Genomic Targets', *Frontiers in Microbiology*, 9. doi: 10.3389/fmicb.2018.02208.

Honorati, M. C. *et al.* (2001) 'High in vivo expression of interleukin-17 receptor in synovial endothelial cells and chondrocytes from arthritis patients', *Rheumatology*. doi: 10.1093/rheumatology/40.5.522.

Hooper, L. V. and Macpherson, A. J. (2010) 'Immune adaptations that maintain homeostasis with the intestinal microbiota', *Nature Reviews Immunology*. Nature Publishing Group, 10(3), pp. 159–169. doi: 10.1038/nri2710.

Horie, M. *et al.* (2016) 'Nonspecific elevation of serum Aspergillus galactomannan antigen levels in patients with rheumatoid arthritis', *Respiratory Investigation*, 54(1), pp. 44–49. doi: 10.1016/j.resinv.2015.08.002.

Horliana, A. C. R. T. *et al.* (2014) 'Dissemination of Periodontal Pathogens in the Bloodstream after Periodontal Procedures: A Systematic Review', *PLoS ONE*. Edited by M. Glogauer. Public Library of Science, 9(5), p. e98271. doi: 10.1371/journal.pone.0098271.

Hornung, B. V. H. *et al.* (2019) 'Response to: "Circulating microbiome in blood of different circulatory compartments" by Schierwagen et al.', *Gut*. BMJ Publishing Group, p. gutjnl-2019-318601. doi: 10.1136/gutjnl-2019-318601.

van der Horst-Bruinsma, I. E. *et al.* (2009) 'A systematic comparison of rheumatoid arthritris and ankylosing spondylitis ', *Clin Exp Rheumatol*, 27, pp. 43–49.

Horta-Baas, G. *et al.* (2017) 'Intestinal Dysbiosis and Rheumatoid Arthritis: A Link between Gut Microbiota and the Pathogenesis of Rheumatoid Arthritis', *Journal of Immunology Research*. Hindawi Limited. doi: 10.1155/2017/4835189.

Horta-Baas, G., … M. R.-F.-J. of immunology and 2017, undefined (no date) 'Intestinal dysbiosis and rheumatoid arthritis: a link between gut microbiota and the pathogenesis of rheumatoid arthritis', *hindawi.com*. Available at: https://www.hindawi.com/journals/jir/2017/4835189/abs/.

Houssiau, F. A. *et al.* (1988) 'Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides', *Arthritis & Rheumatism*. John Wiley & Sons, Ltd, 31(6), pp. 784–788. doi: 10.1002/art.1780310614.

van Houte, J. (1994) 'Role of Micro-organisms in Caries Etiology', *Journal of Dental Research*. SAGE PublicationsSage CA: Los Angeles, CA, 73(3), pp. 672–681. doi: 10.1177/00220345940730031301.

Hovater, M. B. and Sanders, P. W. (2012) 'Effect of Dietary Salt on Regulation of TGF-β in

the Kidney', *Seminars in Nephrology*. doi: 10.1016/j.semnephrol.2012.04.006.

Howson, L. J., Salio, M. and Cerundolo, V. (2015) 'MR1-restricted mucosal-associated invariant T cells and their activation during infectious diseases', *Frontiers in Immunology*. doi: 10.3389/fimmu.2015.00303.

Hrabovský, V. and Siegfried, L. (2009) 'Use of variability of the fungal ITS2 intergenic region for the identification of medically important yeast species', *Section Cellular and Molecular Biology*, 64, pp. 671–675. doi: 10.2478/s11756-009-0120-9.

Huang, A. *et al.* (2006) 'High-throughput identification of clinical pathogenic fungi by hybridization to an oligonucleotide microarray', *Journal of Clinical Microbiology*. American Society for Microbiology Journals, 44(9), pp. 3299–3305. doi: 10.1128/JCM.00417-06.

Huang, C. and Shi, G. (2019) 'Smoking and microbiome in oral, airway, gut and some systemic diseases', *Journal of Translational Medicine*. doi: 10.1186/s12967-019-1971-7.

Huang, Y. J. and Boushey, H. A. (2015) 'The microbiome in asthma', *Journal of Allergy and Clinical Immunology*. Mosby Inc., 135(1), pp. 25–30. doi: 10.1016/j.jaci.2014.11.011.

Huang, Z. *et al.* (2019) 'Antibody neutralization of microbiota-derived circulating peptidoglycan dampens inflammation and ameliorates autoimmunity', *Nature Microbiology*. Nature Publishing Group, 4(5), pp. 766–773. doi: 10.1038/s41564-019-0381-1.

Hugenholtz, F. and de Vos, W. M. (2018) 'Mouse models for human intestinal microbiota research: a critical evaluation', *Cellular and Molecular Life Sciences*. Birkhauser Verlag AG, pp. 149–160. doi: 10.1007/s00018-017-2693-8.

Hutchins, S. S. *et al.* (2015) 'Value of a small control group for estimating intervention effectiveness: Results from simulations of immunization effectiveness studies', *Journal of Comparative Effectiveness Research*. doi: 10.2217/cer.15.11.

Imaoka, A. *et al.* (1996) 'Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ-free mice', *European Journal of Immunology*. John Wiley & Sons, Ltd, 26(4), pp. 945–948. doi: 10.1002/eji.1830260434.

Inan, M. S. *et al.* (2000) 'The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line.', *Gastroenterology*, 118(4), pp. 724–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10734024.

Insenser, M. *et al.* (2018) 'Gut microbiota and the polycystic ovary syndrome: Influence of sex, sex hormones, and obesity', *Journal of Clinical Endocrinology and Metabolism*. doi: 10.1210/jc.2017-02799.

Islander, U. *et al.* (2010) 'Superantigenic Staphylococcus aureus Stimulates Production of Interleukin-17 from Memory but Not Naive T Cells', *Infection and Immunity*, 78(1), pp. 381–386. doi: 10.1128/IAI.00724-09.

Isolauri, E. (2012) 'Development of healthy gut microbiota early in life.', *Journal of paediatrics and child health*, 48 Suppl 3, pp. 1–6. doi: 10.1111/j.1440-1754.2012.02489.x.

Ivanov, I. I. *et al.* (2009a) 'Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria', *Cell*. doi: 10.1016/j.cell.2009.09.033.

Ivanov, I. I. *et al.* (2009b) 'Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria', *Cell*. Cell Press, 139(3), pp. 485–498. doi: 10.1016/J.CELL.2009.09.033.

Ivey, K. L. *et al.* (2011) 'Association between yogurt, milk, and cheese consumption and common carotid artery intima-media thickness and cardiovascular disease risk factors in elderly women', *American Journal of Clinical Nutrition*. doi: 10.3945/ajcn.111.014159.

Jacques, P. *et al.* (2010) 'Invariant Natural Killer T Cells Are Natural Regulators of Murine Spondylarthritis', *Arthritis and Rheumatism*. doi: 10.1002/art.27324.

Jahani-Sherafat, S. *et al.* (2018) 'Role of gut microbiota in the pathogenesis of colorectal cancer; A review article', *Gastroenterology and Hepatology from Bed to Bench*. doi: 10.22037/ghfbb.v0i0.1052.

Jakobsson, H. E. *et al.* (2010) 'Short-term antibiotic treatment has differing long- term impacts on the human throat and gut microbiome', *PLoS ONE*. doi: 10.1371/journal.pone.0009836.

Jan Leipe, H. S.-K. and A. S. (2016) 'IL-22 As a Biomarker for Erosive Disease in Rheumatoid Arthritis - ACR Meeting Abstracts', *Division of Rheumatology and Clinical Immunology, University of Munich, Munich, Germany*. Available at: https://acrabstracts.org/abstract/il-22-as-a-biomarker-for-erosive-disease-in-rheumatoid-arthritis/.

Janda, J. M. and Abbott, S. L. (2007) '16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls.', *Journal of clinical microbiology*. American Society for Microbiology Journals, 45(9), pp. 2761–4. doi: 10.1128/JCM.01228-07.

Jašarević, E., Morrison, K. E. and Bale, T. L. (2016) 'Sex differences in the gut microbiome - Brain axis across the lifespan', *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi: 10.1098/rstb.2015.0122.

Jayasudha, R. *et al.* (2018) 'Alterations in gut bacterial and fungal microbiomes are associated with bacterial Keratitis, an inflammatory disease of the human eye', *Journal of Biosciences*. Springer India, 43(5), pp. 835–856. doi: 10.1007/s12038-018-9798-6.

Jernberg, C. *et al.* (2007) 'Long-term ecological impacts of antibiotic administration on the human intestinal microbiota', *ISME Journal*. doi: 10.1038/ismej.2007.3.

Jiang, Y. *et al.* (2020) 'The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2020.00066.

Jo, J. H., Kennedy, E. A. and Kong, H. H. (2016) 'Research Techniques Made Simple: Bacterial 16S Ribosomal RNA Gene Sequencing in Cutaneous Research', *Journal of Investigative Dermatology*. Elsevier B.V., pp. e23–e27. doi: 10.1016/j.jid.2016.01.005.

Jobin, C. and Sartor, R. B. (2000) 'The IκB/NF-κB system: a key determinant of mucosal inflammation and protection', *American Journal of Physiology-Cell Physiology*. American Physiological SocietyBethesda, MD , 278(3), pp. C451–C462. doi: 10.1152/ajpcell.2000.278.3.C451.

John, G. K. *et al.* (2018) 'Dietary alteration of the gut microbiome and its impact on weight and fat mass: A systematic review and meta-analysis', *Genes*. doi:

10.3390/genes9030167.

Johnson, K. V. A. (2020) 'Gut microbiome composition and diversity are related to human personality traits', *Human Microbiome Journal*. doi: 10.1016/j.humic.2019.100069.

Joossens, M. *et al.* (2011) 'Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives', *Gut*, 60(5), pp. 631–637. doi: 10.1136/gut.2010.223263.

Joosten, L. A. B. and van den Berg, W. B. (2006) 'Murine collagen induced arthritis', in *In Vivo Models of Inflammation*. Birkhäuser Basel, pp. 35–63. doi: 10.1007/978-3-7643-7520-1_2.

Jordan, J. A. and Durso, M. B. (2005) 'Real-Time Polymerase Chain Reaction for Detecting Bacterial DNA Directly from Blood of Neonates Being Evaluated for Sepsis', *The Journal of Molecular Diagnostics*, 7(5), pp. 575–581. doi: 10.1016/S1525-1578(10)60590-9.

Jubair, W. K. *et al.* (2018) 'Modulation of Inflammatory Arthritis in Mice by Gut Microbiota Through Mucosal Inflammation and Autoantibody Generation', *Arthritis and Rheumatology*. John Wiley and Sons Inc., 70(8), pp. 1220–1233. doi: 10.1002/art.40490.

Jung, S. M. *et al.* (2019) 'Sodium chloride aggravates arthritis via Th17 polarization', *Yonsei Medical Journal*. doi: 10.3349/ymj.2019.60.1.88.

Kabir, F. L. *et al.* (2018) 'MicroRNA-145 antagonism reverses TGF-b inhibition of F508del CFTR correction in airway Epithelia', *American Journal of Respiratory and Critical Care Medicine*. doi: 10.1164/rccm.201704-0732OC.

Kalampokis, I., Yoshizaki, A. and Tedder, T. F. (2013) 'IL-10-producing regulatory B cells (B10 cells) in autoimmune disease', *Arthritis Research and Therapy*. doi: 10.1186/ar3907.

Kanki, M. *et al.* (2002) 'Klebsiella pneumoniae produces no histamine: Raoultella planticola and Raoultella ornithinolytica strains are histamine producers.', *Applied and environmental microbiology*. American Society for Microbiology (ASM), 68(7), pp. 3462–6. doi: 10.1128/aem.68.7.3462-3466.2002.

Kanki, P. and Grimes, D. (2012) *Infectious diseases: Selected entries from the encyclopedia of sustainability science and technology*. Available at: https://books.google.co.uk

Kaplan, M. H. (2005) 'STAT4: A Critical Regulator of Inflammation In Vivo', *Immunologic Research*, 31(3), pp. 231–242. doi: 10.1385/IR:31:3:231.

Karlson, E. W. and Deane, K. (2012) 'Environmental and gene-environment interactions and risk of rheumatoid arthritis.', *Rheumatic diseases clinics of North America*. Elsevier, 38(2), pp. 405–26. doi: 10.1016/j.rdc.2012.04.002.

Karunasena, E. *et al.* (2014) 'Host responses to the pathogen Mycobacterium avium subsp. Paratuberculosis and beneficial microbes exhibit host sex specificity', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01229-14.

KASAI, C. *et al.* (2016) 'Comparison of human gut microbiota in control subjects and patients with colorectal carcinoma in adenoma: Terminal restriction fragment length polymorphism and next-generation sequencing analyses', *Oncology Reports*, 35(1), pp.

325–333. doi: 10.3892/or.2015.4398.

Kau, A. L. *et al.* (2015) 'Functional characterization of IgA-targeted bacterial taxa from undernourished Malawian children that produce diet-dependent enteropathy', *Science translational medicine*. doi: 10.1126/scitranslmed.aaa4877.

KAWAGUCHI-MIYASHITA, M. *et al.* (1996) 'Development and cytolytic function of intestinal intraepithelial T lymphocytes in antigen-minimized mice', *Immunology*. John Wiley & Sons, Ltd (10.1111), 89(2), pp. 268–273. doi: 10.1046/j.1365-2567.1996.d01-740.x.

Kawai, T. and Akira, S. (2010) 'The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors', *Nature Immunology*. Nature Publishing Group, 11(5), pp. 373–384. doi: 10.1038/ni.1863.

KAWASAKI, T. *et al.* (2016) 'Allergic bronchopulmonary aspergillosis in a patient with rheumatoid arthritis under adalimumab therapy: a case report', *Japanese Journal of Clinical Immunology*, 39(1), pp. 84–89. doi: 10.2177/jsci.39.84.

Kell, D. B. and Pretorius, E. (2015) 'On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: the central roles of LPS and LPS-induced cell death', *Integrative Biology*. Narnia, 7(11), pp. 1339–1377. doi: 10.1039/c5ib00158g.

Kempsell, K. E. *et al.* (2000) 'Reverse transcriptase-PCR analysis of bacterial rRNA for detection and characterization of bacterial species in arthritis synovial tissue', *Infection and Immunity*. doi: 10.1128/IAI.68.10.6012-6026.2000.

Kempsell, Karen E. *et al.* (2000) 'Reverse Transcriptase-PCR Analysis of Bacterial rRNA for Detection and Characterization of Bacterial Species in Arthritis Synovial Tissue', *Infection and Immunity*. American Society for Microbiology Journals, 68(10), pp. 6012–6026. doi: 10.1128/IAI.68.10.6012-6026.2000.

Kenna, T. J. *et al.* (2012) 'Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive γ/δ T cells in patients with active ankylosing spondylitis', *Arthritis & Rheumatism*, 64(5), pp. 1420–1429. doi: 10.1002/art.33507.

Khan, T. J. *et al.* (2019) 'Association of gut dysbiosis with intestinal metabolites in response to antibiotic treatment', *Human Microbiome Journal*. doi: 10.1016/j.humic.2018.11.004.

Khanna, A., Khanna, M. and Aggarwal, A. (2013) 'Serratia Marcescens- A Rare Opportunistic Nosocomial Pathogen and Measures to Limit its Spread in Hospitalized Patients', *Journal of Clinical and Diagnostic Research : JCDR*. JCDR Research & Publications Private Limited, 7(2), p. 243. doi: 10.7860/JCDR/2013/5010.2737.

Kho, Z. Y. and Lal, S. K. (2018) 'The human gut microbiome - A potential controller of wellness and disease', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.01835.

Kim, H.-R. *et al.* (2007) 'Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF- B- and p38 MAPK-dependent signalling pathways', *Rheumatology*, 46(1), pp. 57–64. doi: 10.1093/rheumatology/kel159.

Kim, H. -R. *et al.* (2007) 'The clinical role of IL-23p19 in patients with rheumatoid arthritis',

Scandinavian Journal of Rheumatology. Taylor & Francis, 36(4), pp. 259–264. doi: 10.1080/03009740701286813.

Kim, J. H. *et al.* (2011) 'A Case of Invasive Pulmonary Aspergillosis in a Patient with Rheumatoid Arthritis Treated with Adalimumab', *Journal of Rheumatic Diseases*. The Korean Rheumatism Association (KAMJE), 18(3), p. 212. doi: 10.4078/jrd.2011.18.3.212.

Kim, K.-W. *et al.* (2017) 'Histamine and Histamine H4 Receptor Promotes Osteoclastogenesis in Rheumatoid Arthritis', *Scientific Reports*, 7(1), p. 1197. doi: 10.1038/s41598-017-01101-y.

Kim, K. K., Lee, J.-S. and Stevens, D. A. (2013) 'Microbiology and epidemiology of Halomonas species', *Future Microbiology*. Future Medicine Ltd London, UK, 8(12), pp. 1559–1573. doi: 10.2217/fmb.13.108.

Kim, M. *et al.* (2016) 'Gut Microbial Metabolites Fuel Host Antibody Responses', *Cell Host and Microbe*. doi: 10.1016/j.chom.2016.07.001.

Kim, Y. S. *et al.* (2020) 'Sex differences in gut microbiota', *World Journal of Men?s Health*. Korean Society for Sexual Medicine and Andrology, pp. 48–60. doi: 10.5534/wjmh.190009.

Kinane, D. F. *et al.* (2005) 'Bacteraemia following periodontal procedures', *Journal of Clinical Periodontology*, 32(7), pp. 708–713. doi: 10.1111/j.1600-051X.2005.00741.x.

Kinne, Raimund W. *et al.* (2000) 'Macrophages in rheumatoid arthritis', *Arthritis Research*, pp. 189–202. doi: 10.1186/ar86.

Kinne, Raimund W *et al.* (2000) 'Macrophages in rheumatoid arthritis', *Arthritis Research*. BioMed Central, 2(3), p. 189. doi: 10.1186/ar86.

Kishimoto, T. (1992) 'Interleukin-6 and its receptor in autoimmunity', *Journal of Autoimmunity*. Academic Press, 5, pp. 123–132. doi: 10.1016/0896-8411(92)90027-N.

Kjeldsen-Kragh, J. *et al.* (1991) 'Controlled trial of fasting and one-year vegetarian diet in rheumatoid arthritis', *The Lancet*. doi: 10.1016/0140-6736(91)91770-U.

Klein, S. L. and Flanagan, K. L. (2016) 'Sex differences in immune responses', *Nature Reviews Immunology*. doi: 10.1038/nri.2016.90.

Klingberg, E. *et al.* (2019) 'Weight loss improves disease activity in patients with psoriatic arthritis and obesity: An interventional study', *Arthritis Research and Therapy*. doi: 10.1186/s13075-019-1810-5.

Kneitz, C., Wilhelm, M. and Tony, H. P. (2004) 'Improvement of refractory rheumatoid arthritis after depletion of B cells', *Scandinavian Journal of Rheumatology*, 33(2), pp. 82–86. doi: 10.1080/03009740310004379.

Kobak, S. *et al.* (2014) 'Severe candida laryngitis in a patient with rheumatoid arthritis treated with adalimumab', *European Journal of Rheumatology*. doi: 10.5152/eurjrheumatol.2014.140001.

Kobayashi, N. *et al.* (2019) 'The Roles of Peyer's Patches and Microfold Cells in the Gut Immune System: Relevance to Autoimmune Diseases', *Frontiers in Immunology*. Frontiers Media S.A. doi: 10.3389/fimmu.2019.02345.

Kobori, A. *et al.* (2010) 'Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis', *Journal of Gastroenterology*. doi: 10.1007/s00535-010-0245-1.

Koch, M. A. *et al.* (2016) 'Maternal IgG and IgA Antibodies Dampen Mucosal T Helper Cell Responses in Early Life', *Cell*. doi: 10.1016/j.cell.2016.04.055.

Koeth, R. A. *et al.* (2013) 'Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis', *Nature Medicine*. doi: 10.1038/nm.3145.

Kohno, M. *et al.* (2008) 'Interleukin-17 gene expression in patients with rheumatoid arthritis', *Modern Rheumatology*, 18(1), pp. 15–22. doi: 10.3109/s10165-007-0015-y.

Kolwijck, E. and van de Veerdonk, F. L. (2014) 'The potential impact of the pulmonary microbiome on immunopathogenesis of *Aspergillus* -related lung disease', *European Journal of Immunology*. John Wiley & Sons, Ltd, 44(11), pp. 3156–3165. doi: 10.1002/eji.201344404.

Komatsu, N. and Takayanagi, H. (2012) 'Inflammation and Bone Destruction in Arthritis: Synergistic Activity of Immune and Mesenchymal Cells in Joints', *Frontiers in Immunology*. Frontiers, 3, p. 77. doi: 10.3389/fimmu.2012.00077.

Komatsu, N. and Takayanagi, H. (2018) 'Immune-bone interplay in the structural damage in rheumatoid arthritis', *Clinical & Experimental Immunology*. Blackwell Publishing Ltd, 194(1), pp. 1–8. doi: 10.1111/cei.13188.

Korn, T. *et al.* (2009) 'IL-17 and Th17 Cells', *Annual Review of Immunology*, 27(1), pp. 485–517. doi: 10.1146/annurev.immunol.021908.132710.

Koslowski, M. J. *et al.* (2010) 'Innate antimicrobial host defense in small intestinal Crohn's disease', *International Journal of Medical Microbiology*. Urban & Fischer, 300(1), pp. 34–40. doi: 10.1016/J.IJMM.2009.08.011.

Kostic, A. D., Howitt, M. R. and Garrett, W. S. (2013) 'Exploring host-microbiota interactions in animal models and humans', *Genes and Development*. doi: 10.1101/gad.212522.112.

Kotake, S. *et al.* (1999) 'IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis', *Journal of Clinical Investigation*. doi: 10.1172/JCI5703.

Kotake, S. *et al.* (2009) 'Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation', *Journal of Bone and Mineral Research*. John Wiley & Sons, Ltd, 11(1), pp. 88–95. doi: 10.1002/jbmr.5650110113.

Koziel, J., Mydel, P. and Potempa, J. (2014) 'The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review', *Current Rheumatology Reports*, 16(3), p. 408. doi: 10.1007/s11926-014-0408-9.

Kozik, A. J. *et al.* (2017) 'Age, sex, and TNF associated differences in the gut microbiota of mice and their impact on acute TNBS colitis', *Experimental and Molecular Pathology*. doi: 10.1016/j.yexmp.2017.11.014.

Kriaa, A. *et al.* (2019) 'Microbial impact on cholesterol and bile acid metabolism: current status and future prospects.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 60(2), pp. 323–332. doi: 10.1194/jlr.R088989.

Krych, Ł. *et al.* (2015) 'Gut microbial markers are associated with diabetes onset, regulatory imbalance, and IFN-γ level in NOD Mice', *Gut Microbes*. Taylor & Francis, 6(2), pp. 101–109. doi: 10.1080/19490976.2015.1011876.

Kubinak, J. L. *et al.* (2015) 'MHC variation sculpts individualized microbial communities that control susceptibility to enteric infection', *Nature Communications*. doi: 10.1038/ncomms9642.

Kwon, O. G. *et al.* (2014) 'The effects of platycodin D, a saponin purified from platycodi radix, on collagen-induced DBA/1J mouse rheumatoid arthritis', *Evidence-based Complementary and Alternative Medicine*. doi: 10.1155/2014/954508.

Lagkouvardos, I. *et al.* (2016) 'IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies', *Scientific Reports*. Nature Publishing Group, 6(1), p. 33721. doi: 10.1038/srep33721.

Lam, P. W. and Salit, I. E. (2014) 'Raoultella planticola bacteremia following consumption of seafood.', *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale*. Hindawi Limited, 25(4), pp. e83-4.

Langdon, A., Crook, N. and Dantas, G. (2016) 'The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation', *Genome Medicine*. BioMed Central Ltd. doi: 10.1186/s13073-016-0294-z.

Laria, A. *et al.* (2016) 'The macrophages in rheumatic diseases', *Journal of Inflammation Research*. Dove Medical Press Ltd, pp. 1–11. doi: 10.2147/JIR.S82320.

Lauder, A. P. *et al.* (2016a) 'Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota', *Microbiome*. BioMed Central, 4(1), p. 29. doi: 10.1186/s40168-016-0172-3.

Lauder, A. P. *et al.* (2016b) 'Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota', *Microbiome*. BioMed Central, 4(1), p. 29. doi: 10.1186/s40168-016-0172-3.

Laurence, M., Hatzis, C. and Brash, D. E. (2014) 'Common Contaminants in Next-Generation Sequencing That Hinder Discovery of Low-Abundance Microbes', *PLoS ONE*. Edited by T. Gilbert, 9(5), p. e97876. doi: 10.1371/journal.pone.0097876.

Lécuyer, E. *et al.* (2014) 'Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses', *Immunity*. doi: 10.1016/j.immuni.2014.03.009.

Lee, J. Y. *et al.* (2017) 'Probiotics reduce repeated water avoidance stress-induced colonic microinflammation in Wistar rats in a sex-specific manner', *PLoS ONE*. doi: 10.1371/journal.pone.0188992.

Lee, Y. K. *et al.* (2011) 'Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis.', *Proceedings of the National Academy of*

Sciences of the United States of America. National Academy of Sciences, 108 Suppl(Supplement 1), pp. 4615–22. doi: 10.1073/pnas.1000082107.

Lefèvre, S. *et al.* (2009) 'Synovial fibroblasts spread rheumatoid arthritis to unaffected joints', *Nature Medicine*, 15(12), pp. 1414–1420. doi: 10.1038/nm.2050.

Leipe, J. *et al.* (2011) 'Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis.', *Annals of the rheumatic diseases*. BMJ Publishing Group Ltd, 70(8), pp. 1453–7. doi: 10.1136/ard.2011.152074.

Levinson, W. (2014) *Review of Medical Microbiology and Immunology*, *The effects of brief mindfulness intervention on acute pain experience: An examination of individual difference*. doi: 10.1017/CBO9781107415324.004.

Levorova, J. *et al.* (2017) 'Septic arthritis of the temporomandibular joint caused by rare bacteria Raoultella ornithinolytica', *International Journal of Oral and Maxillofacial Surgery*, 46(1), pp. 111–115. doi: 10.1016/j.ijom.2016.09.008.

Ley, R. E. *et al.* (2005) 'Obesity alters gut microbial ecology', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0504978102.

Ley, R. E. (2010) 'Obesity and the human microbiome', *Current Opinion in Gastroenterology*, 26(1), pp. 5–11. doi: 10.1097/MOG.0b013e328333d751.

Li, M. *et al.* (2008) 'Symbiotic gut microbes modulate human metabolic phenotypes', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0712038105.

Li, W. *et al.* (2014) 'Lipopolysaccharide-induced profiles of cytokine, chemokine, and growth factors produced by human decidual cells are altered by lactobacillus rhamnosus gr-1 supernatant', *Reproductive Sciences*. doi: 10.1177/1933719113519171.

Liao, T. *et al.* (2017) 'Chondroitin Sulfate Flourishes Gut Sulfatase-Secreting Bacteria To Damage Mucus Layers, Leak Bacterial Debris, And Trigger Inflammatory Lesions In Mice', *bioRxiv*. Cold Spring Harbor Laboratory, p. 145714. doi: 10.1101/145714.

Lim, H. *et al.* (2018) 'Structural biology of the TNFα antagonists used in the treatment of rheumatoid arthritis', *International Journal of Molecular Sciences*. doi: 10.3390/ijms19030768.

Lim, Y. *et al.* (2017) 'Oral Microbiome: A New Biomarker Reservoir for Oral and Oropharyngeal Cancers', *Theranostics*, 7(17), pp. 4313–4321. doi: 10.7150/thno.21804.

Limon, J. J., Skalski, J. H. and Underhill, D. M. (2017) 'Commensal Fungi in Health and Disease', *Cell Host & Microbe*, 22(2), pp. 156–165. doi: 10.1016/j.chom.2017.07.002.

Lin, P. *et al.* (2014) 'HLA-B27 and Human b2-Microglobulin Affect the Gut Microbiota of Transgenic Rats', *PLoS ONE*, 9(8), p. 105684. doi: 10.1371/journal.pone.0105684.

Lindqvist, E *et al.* (2005) 'Prognostic laboratory markers of joint damage in rheumatoid arthritis', *Ann Rheum Dis*, 64, pp. 196–201. doi: 10.1136/ard.2003.019992.

Liu, C. M. *et al.* (2015) 'Staphylococcus aureus and the ecology of the nasal microbiome', *Science Advances*. American Association for the Advancement of Science, 1(5), p.

e1400216. doi: 10.1126/sciadv.1400216.

Liu, D. (2011) *Molecular detection of human fungal pathogens*. CRC Press. Available at: https://books.google.co.uk/books.

Liu, J. *et al.* (2019) 'Beneficial effects of tea water extracts on the body weight and gut microbiota in C57BL/6J mice fed with a high-fat diet', *Food and Function*. Royal Society of Chemistry, 10(5), pp. 2847–2860. doi: 10.1039/c8fo02051e.

Liu, S. (ed.) (2018) *Rheumatoid Arthritis*. New York, NY: Springer New York (Methods in Molecular Biology). doi: 10.1007/978-1-4939-8802-0.

Liu, W. *et al.* (2015) 'Elevated serum levels of IL-6 and IL-17 may associate with the development of ankylosing spondylitis.', *International journal of clinical and experimental medicine*, 8(10), pp. 17362–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26770328.

Liu, X. *et al.* (2016) 'Role of the Gut Microbiome in Modulating Arthritis Progression in Mice', *Scientific Reports*. Nature Publishing Group, 6(1), p. 30594. doi: 10.1038/srep30594.

Lockhart, E., Green, A. M. and Flynn, J. L. (2006) 'IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 177(7), pp. 4662–9. doi: 10.4049/JIMMUNOL.177.7.4662.

Löe, H., Theilade, E. and Jensen, S. B. (1965) 'Experimental Gingivitis in Man', *Journal of Periodontology*, 36(3), pp. 177–187. doi: 10.1902/jop.1965.36.3.177.

López-Menduiña, M. *et al.* (2010) 'Systemic IGF-I administration attenuates the inhibitory effect of chronic arthritis on gastrocnemius mass and decreases atrogin-1 and IGFBP-3', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*. doi: 10.1152/ajpregu.00211.2010.

Lopez-Oliva, I. *et al.* (2018) 'Dysbiotic Subgingival Microbial Communities in Periodontally Healthy Patients With Rheumatoid Arthritis', *Arthritis & Rheumatology*, 70(7), pp. 1008–1013. doi: 10.1002/art.40485.

Lorenz, M. G., Mühl, H. and Disqué, C. (2015) 'Bacterial and Fungal DNA Extraction from Blood Samples: Manual Protocols', in, pp. 109–119. doi: 10.1007/978-1-4939-1776-1_11.

Loyola-Rodriguez, J. P. *et al.* (2010) 'Rheumatoid arthritis and the role of oral bacteria.', *Journal of oral microbiology*. Taylor & Francis, 2. doi: 10.3402/jom.v2i0.5784.

Lozupone, C. A. *et al.* (2013) 'Meta-analyses of studies of the human microbiota', *Genome Research*, 23(10), pp. 1704–1714. doi: 10.1101/gr.151803.112.

Lubberts, E. (2010) 'Th17 cytokines and arthritis', *Seminars in Immunopathology*. Springer-Verlag, 32(1), pp. 43–53. doi: 10.1007/s00281-009-0189-9.

Lundberg, K. *et al.* (2008) 'Antibodies to citrullinated α-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase', *Arthritis & Rheumatism*. John Wiley & Sons, Ltd, 58(10), pp. 3009–3019. doi: 10.1002/art.23936.

Lundy, S. K. *et al.* (2007) 'Cells of the synovium in rheumatoid arthritis. T lymphocytes',

Arthritis Research & Therapy. BioMed Central, 9(1), p. 202. doi: 10.1186/ar2107.

Luo, X. M. *et al.* (2018) 'Gut Microbiota in Human Systemic Lupus Erythematosus and a Mouse Model of Lupus.', *Applied and environmental microbiology*. American Society for Microbiology (ASM), 84(4). doi: 10.1128/AEM.02288-17.

Lyte, M. *et al.* (2016) 'Resistant starch alters the microbiota-gut brain axis: Implications for dietary modulation of behavior', *PLoS ONE*. doi: 10.1371/journal.pone.0146406.

Mackay, I. R. and Rose, N. R. (2014) *The Autoimmune Diseases*. Elsevier/Academic Press.

Macpherson, A. J., Geuking, M. B. and McCoy, K. D. (2005) 'Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria', *Immunology*. John Wiley & Sons, Ltd (10.1111), 115(2), pp. 153–162. doi: 10.1111/j.1365-2567.2005.02159.x.

Macpherson, A. J. and Uhr, T. (2004) 'Induction of Protective IgA by Intestinal Dendritic Cells Carrying Commensal Bacteria', *Science*. doi: 10.1126/science.1091334.

De Madaria, E. *et al.* (2005) 'Detection and identification of bacterial DNA in serum from patients with acute pancreatitis', *Gut*. doi: 10.1136/gut.2004.047514.

Maeda, Y. *et al.* (2016) 'Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine', *Arthritis & Rheumatology*. John Wiley & Sons, Ltd, 68(11), pp. 2646–2661. doi: 10.1002/art.39783.

Maeda, Y. and Takeda, K. (2017) 'Role of Gut Microbiota in Rheumatoid Arthritis.', *Journal of clinical medicine*. Multidisciplinary Digital Publishing Institute (MDPI), 6(6). doi: 10.3390/jcm6060060.

Magari, K. *et al.* (2004) 'Inflammatory cytokine levels in paw tissues during development of rat collagen-induced arthritis: Effect of FK506, an inhibitor of T cell activation', *Inflammation Research*. doi: 10.1007/s00011-004-1284-y.

Maharaj, B., Coovadia, Y. and Vayej, A. C. (2012) 'An investigation of the frequency of bacteraemia following dental extraction, tooth brushing and chewing.', *Cardiovascular journal of Africa*. Clinics Cardive Publishing (Pty) Ltd., 23(6), pp. 340–4. doi: 10.5830/CVJA-2012-016.

Makala, L. H. C., Suzuki, N. and Nagasawa, H. (2002) 'Peyer's patches: Organized lymphoid structures for the induction of mucosal immune responses in the intestine', *Pathobiology*, pp. 55–68. doi: 10.1159/000067305.

Maloy, K. J. and Powrie, F. (2011) 'Intestinal homeostasis and its breakdown in inflammatory bowel disease', *Nature*. Nature Publishing Group, 474(7351), pp. 298–306. doi: 10.1038/nature10208.

Manasson, J. *et al.* (2020) 'Interleukin-17 Inhibition in Spondyloarthritis Is Associated With Subclinical Gut Microbiome Perturbations and a Distinctive Interleukin-25–Driven Intestinal Inflammation', *Arthritis and Rheumatology*. doi: 10.1002/art.41169.

Manasson, J., Blank, R. B. and Scher, J. U. (2020) 'The microbiome in rheumatology: Where are we and where should we go?', *Annals of the Rheumatic Diseases*. BMJ Publishing Group, pp. 727–733. doi: 10.1136/annrheumdis-2019-216631.

Mancabelli, L. *et al.* (2017) 'Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis', *FEMS Microbiology Ecology*. Narnia, 93(12). doi: 10.1093/femsec/fix153.

Mantis, N. J., Rol, N. and Corthésy, B. (2011) 'Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut', *Mucosal Immunology*. doi: 10.1038/mi.2011.41.

Manz, M., Beglinger, C. and Vavricka, S. R. (2009) 'Fatal invasive pulmonary aspergillosis associated with adalimumab therapy.', *Gut*. BMJ Publishing Group, 58(1), p. 149. doi: 10.1136/gut.2008.161638.

Mar, J. S. *et al.* (2016) 'Disease severity and immune activity relate to distinct interkingdom gut microbiome states in ethnically distinct ulcerative colitis patients', *mBio*. American Society for Microbiology, 7(4). doi: 10.1128/mBio.01072-16.

Marchesi, J. R. and Ravel, J. (2015) 'The vocabulary of microbiome research: a proposal', *Microbiome*. BioMed Central, 3(1), p. 31. doi: 10.1186/s40168-015-0094-5.

Margolis, K. L. *et al.* (2011) 'A Diet High in Low-Fat Dairy Products Lowers Diabetes Risk in Postmenopausal Women', *The Journal of Nutrition*. doi: 10.3945/jn.111.143339.

Marinova-Mutafchieva, L. *et al.* (1997) 'Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA)', *Clinical and Experimental Immunology*. doi: 10.1046/j.1365-2249.1997.2901181.x.

Markova, N. D. (2017) *L-form bacteria cohabitants in human blood: significance for health and diseases*. Discovery Medicine. doi: . Discov Med 23, 305-313.

Marshall, J. S. *et al.* (2018) 'An introduction to immunology and immunopathology', *Allergy, Asthma and Clinical Immunology*. BioMed Central Ltd., p. 49. doi: 10.1186/s13223-018-0278-1.

Martín, A. I. *et al.* (2008) 'Adipose tissue loss in adjuvant arthritis is associated with a decrease in lipogenesis, but not with an increase in lipolysis', *Journal of Endocrinology*. doi: 10.1677/JOE-07-0491.

Martin, B. *et al.* (2009) 'Interleukin-17-Producing γδ T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals', *Immunity*. doi: 10.1016/j.immuni.2009.06.020.

Martin, C. R. *et al.* (2018) 'The Brain-Gut-Microbiome Axis', *CMGH*. doi: 10.1016/j.jcmgh.2018.04.003.

Martin, R. *et al.* (2016) 'Early-Life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota', *PLoS ONE*. doi: 10.1371/journal.pone.0158498.

Martínez-Cánovas, M. J. *et al.* (2004) 'Halomonas ventosae sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium', *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijs.0.02942-0.

MARTÍNEZ-GONZÁLEZ, O. *et al.* (1994) 'INTESTINAL PERMEABILITY IN PATIENTS WITH ANKYLOSING SPONDYLITIS AND THEIR HEALTHY RELATIVES', *Rheumatology*. Oxford University Press, 33(7), pp. 644–647. doi: 10.1093/rheumatology/33.7.644.

Martinez-Martinez, R. E. *et al.* (2009) 'Detection of periodontal bacterial DNA in serum and synovial fluid in refractory rheumatoid arthritis patients', *Journal of Clinical Periodontology*, 36(12), pp. 1004–1010. doi: 10.1111/j.1600-051X.2009.01496.x.

Maslowski, K. M. and Mackay, C. R. (2011) 'Diet, gut microbiota and immune responses', *Nature Immunology*, 12(1), pp. 5–9. doi: 10.1038/ni0111-5.

Matsumoto, T., Tsurumoto, T. and Shindo, H. (2006) 'Interleukin-6 levels in synovial fluids of patients with rheumatoid arthritis correlated with the infiltration of inflammatory cells in synovial membrane', *Rheumatology International*, 26(12), pp. 1096–1100. doi: 10.1007/s00296-006-0143-2.

Mazmanian, S. K. *et al.* (2005) 'An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System', *Cell*. Cell Press, 122(1), pp. 107–118. doi: 10.1016/J.CELL.2005.05.007.

McBeth, A. and Dobner, P. (2019) 'Fecal Transplant: How an Ancient Therapy Is Finding a New Use in Today's Antibiotic-Resistant Era', *Journal of Restorative Medicine*. doi: 10.14200/jrm.2019.0117.

Mcdonald, C. and Nunez, G. (2005) 'NOD-LRR proteins: Role in host-microbial interactions and inflammatory disease', *Article in Annual Review of Biochemistry*. Annual Reviews, 74(1), pp. 355–383. doi: 10.1146/annurev.biochem.74.082803.133347.

Mcilroy, J. R. *et al.* (2019) 'Current and future targets for faecal microbiota transplantation', *Human Microbiome Journal*. doi: 10.1016/j.humic.2018.08.004.

McInnes, I. B., Buckley, C. D. and Isaacs, J. D. (2016) 'Cytokines in rheumatoid arthritis — shaping the immunological landscape', *Nature Reviews Rheumatology*, 12(1), pp. 63–68. doi: 10.1038/nrrheum.2015.171.

McInnes, I. B. and Schett, G. (2011) 'The Pathogenesis of Rheumatoid Arthritis', *New England Journal of Medicine*, 365(23), pp. 2205–2219. doi: 10.1056/NEJMra1004965.

Mease, P. *et al.* (2019) 'Safety of Ixekizumab in Patients With Psoriatic Arthritis: Results From a Pooled Analysis of Three Clinical Trials', *Arthritis Care and Research*. doi: 10.1002/acr.23738.

Mease, P. J. (2015) 'Inhibition of interleukin-17, interleukin-23 and the TH17 cell pathway in the treatment of psoriatic arthritis and psoriasis', *Current Opinion in Rheumatology*. doi: 10.1097/BOR.0000000000000147.

Meednu, N. *et al.* (2016) 'Production of RANKL by Memory B Cells: A Link Between B Cells and Bone Erosion in Rheumatoid Arthritis', *Arthritis & Rheumatology*, 68(4), pp. 805–816. doi: 10.1002/art.39489.

Mei, Y. *et al.* (2011) 'Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis', *Clinical Rheumatology*, 30(2), pp. 269–273. doi: 10.1007/s10067-010-1647-4.

Melis, L. *et al.* (2010) 'Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis', *Annals of the Rheumatic Diseases*. BMJ Publishing Group Ltd, 69(3), pp. 618–623. doi: 10.1136/ARD.2009.107649.

Merola, J. F., Espinoza, L. R. and Fleischmann, R. (2018) 'Distinguishing rheumatoid

arthritis from psoriatic arthritis Psoriatic arthritis', *RMD Open*, 4, p. 656. doi: 10.1136/rmdopen-2018-000656.

Metawi, S. A. *et al.* (2011) 'Serum and synovial fluid levels of interleukin-17 in correlation with disease activity in patients with RA', *Clinical Rheumatology*. Springer-Verlag, 30(9), pp. 1201–1207. doi: 10.1007/s10067-011-1737-y.

Michalovich, D. *et al.* (2019) 'Obesity and disease severity magnify disturbed microbiome-immune interactions in asthma patients', *Nature Communications*. Nature Research, 10(1), pp. 1–14. doi: 10.1038/s41467-019-13751-9.

Mielants, H. *et al.* (1992) 'Increased intestinal permeability in ankylosing spondylitis.', *Gut*. BMJ Publishing Group, 33(8), p. 1150. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1398245.

Miettinen, M., Vuopio-Varkila, J. and Varkila, K. (1996) 'Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria.', *Infection and immunity*. American Society for Microbiology (ASM), 64(12), pp. 5403–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8945595.

Mikkelsen, K. H., Allin, K. H. and Knop, F. K. (2016) 'Effect of antibiotics on gut microbiota, glucose metabolism and body weight regulation: A review of the literature', *Diabetes, Obesity and Metabolism*. doi: 10.1111/dom.12637.

Mikuls, T. R. *et al.* (2018) 'The subgingival microbiome in patients with established rheumatoid arthritis', *Rheumatology*, 57(7), pp. 1162–1172. doi: 10.1093/rheumatology/key052.

Milani, C. *et al.* (2017) 'The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota', *Microbiol. Mol. Biol. Rev.* American Society for Microbiology, 81(4), pp. e00036-17. doi: 10.1128/MMBR.00036-17.

Di Minno, M. N. D. *et al.* (2014) 'Weight loss and achievement of minimal disease activity in patients with psoriatic arthritis starting treatment with tumour necrosis factor α blockers', *Annals of the Rheumatic Diseases*. doi: 10.1136/annrheumdis-2012-202812.

Moayyedi, P. *et al.* (2015) 'Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial', *Gastroenterology*. doi: 10.1053/j.gastro.2015.04.001.

Moen, K. *et al.* (2003) 'Immunoglobulin G and A antibody responses to Bacteroides forsythus and Prevotella intermedia in sera and synovial fluids of arthritis patients.', *Clinical and diagnostic laboratory immunology*. American Society for Microbiology, 10(6), pp. 1043–50. doi: 10.1128/CDLI.10.6.1043-1050.2003.

Moen, K. *et al.* (2005) 'Oral bacterial DNAs in synovial fluids of arthritis patients', *Microbial Ecology in Health and Disease*. Taylor & Francis, 17(1), pp. 2–8. doi: 10.1080/08910600510031394.

Moen, K. *et al.* (2006) 'Synovial inflammation in active rheumatoid arthritis and psoriatic arthritis facilitates trapping of a variety of oral bacterial DNAs.', *Clinical and experimental rheumatology*, 24(6), pp. 656–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17207381.

Mohammadi, T. *et al.* (2005) 'Removal of contaminating DNA from commercial nucleic acid extraction kit reagents', *Journal of Microbiological Methods*. Elsevier, 61(2), pp. 285–288. doi: 10.1016/J.MIMET.2004.11.018.

Mohammed, R. H. A. and Elmakhzangy, H. I. I. (2017) 'The Gut Microbiota and Inflammation in Rheumatoid Arthritis', in *New Developments in the Pathogenesis of Rheumatoid Arthritis*. InTech. doi: 10.5772/67368.

Mokuno, Y. *et al.* (2000) 'Expression of Toll-Like Receptor 2 on T Cells Bearing Invariant V 6/V 1 Induced by Escherichia coli Infection in Mice', *The Journal of Immunology*, 165(2), pp. 931–940. doi: 10.4049/jimmunol.165.2.931.

Morita, T. *et al.* (2016) 'The proportion of regulatory T cells in patients with rheumatoid arthritis: A meta-Analysis', *PLoS ONE*. Public Library of Science, 11(9). doi: 10.1371/journal.pone.0162306.

Morris, A. J. *et al.* (1991) 'Increased intestinal permeability in ankylosing spondylitis--primary lesion or drug effect?', *Gut*. BMJ Publishing Group, 32(12), pp. 1470–2. doi: 10.1136/GUT.32.12.1470.

Morton, A. M. *et al.* (2014) 'Endoscopic photoconversion reveals unexpectedly broad leukocyte trafficking to and from the gut.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(18), pp. 6696–701. doi: 10.1073/pnas.1405634111.

Mueller, S. *et al.* (2006) 'Differences in fecal microbiota in different European study populations in relation to age, gender, and country: A cross-sectional study', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.72.2.1027-1033.2006.

van den Munckhof, I. C. L. *et al.* (2018) 'Role of gut microbiota in chronic low-grade inflammation as potential driver for atherosclerotic cardiovascular disease: a systematic review of human studies', *Obesity Reviews*, 19(12), pp. 1719–1734. doi: 10.1111/obr.12750.

Na, H. S. *et al.* (2017) 'Th17 and IL-17 Cause Acceleration of Inflammation and Fat Loss by Inducing α2-Glycoprotein 1 (AZGP1) in Rheumatoid Arthritis with High-Fat Diet', *American Journal of Pathology*. doi: 10.1016/j.ajpath.2016.12.023.

Nagpal, R., Wang, S., *et al.* (2018) 'Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.02897.

Nagpal, R., Mainali, R., *et al.* (2018) 'Gut microbiome and aging: Physiological and mechanistic insights', *Nutrition and Healthy Aging*. doi: 10.3233/NHA-170030.

Nakasone, E. S. *et al.* (2015) 'Emergence of Raoultella ornithinolytica on O'ahu: a case of community-acquired R. ornithinolytica urinary tract infection.', *Hawai'i journal of medicine & public health : a journal of Asia Pacific Medicine & Public Health*, 74(5), pp. 174–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26019987.

Narazaki, M., Tanaka, T. and Kishimoto, T. (2017a) 'The role and therapeutic targeting of IL-6 in rheumatoid arthritis', *Expert Review of Clinical Immunology*, 13(6), pp. 535–551. doi: 10.1080/1744666X.2017.1295850.

Narazaki, M., Tanaka, T. and Kishimoto, T. (2017b) 'The role and therapeutic targeting of IL-6 in rheumatoid arthritis', *Expert Review of Clinical Immunology*. Taylor & Francis, 13(6), pp. 535–551. doi: 10.1080/1744666X.2017.1295850.

Nayak, R. R. *et al.* (2019) 'Perturbation of the human gut microbiome by a non-antibiotic drug contributes to the resolution of autoimmune disease', *bioRxiv*. doi: 10.1101/600155.

Neutra, M. R., Frey, A. and Kraehenbuhl, J. P. (1996) 'Epithelial M cells: Gateways for mucosal infection and immunization', *Cell*. Cell Press, pp. 345–348. doi: 10.1016/S0092-8674(00)80106-3.

Nguyen, T. L. A. *et al.* (2015) 'How informative is the mouse for human gut microbiota research?', *DMM Disease Models and Mechanisms*. doi: 10.1242/dmm.017400.

Nibali, L. and Henderson, B. (2016) *The human microbiota and chronic disease: Dysbiosis as a cause of human pathology*, *The Human Microbiota and Chronic Disease: Dysbiosis as a Cause of Human Pathology*. doi: 10.1002/9781118982907.

Nica, M. *et al.* (2010) *MOLECULAR DETECTION AND IDENTIFICATION OF PATHOGENIC FUNGI IN CLINICAL SAMPLES*. Available at: www.iata.org.

NIENHUIS, R. L. and MANDEMA, E. (1964) 'A NEW SERUM FACTOR IN PATIENTS WITH RHEUMATOID ARTHRITIS; THE ANTIPERINUCLEAR FACTOR.', *Annals of the rheumatic diseases*. doi: 10.1136/ard.23.4.302.

Nikkari, S. *et al.* (2001) 'Does blood of healthy subjects contain bacterial ribosomal DNA?', *Journal of clinical microbiology*. American Society for Microbiology Journals, 39(5), pp. 1956–9. doi: 10.1128/JCM.39.5.1956-1959.2001.

Ogata, A., Kumanogoh, A. and Tanaka, T. (2012) 'Pathological role of interleukin-6 in psoriatic arthritis.', *Arthritis*. Hindawi Limited, 2012, p. 713618. doi: 10.1155/2012/713618.

Ogrendik, M. (2009) 'Rheumatoid arthritis is linked to oral bacteria: etiological association', *Modern Rheumatology*. Springer Japan, 19(5), pp. 453–456. doi: 10.1007/s10165-009-0194-9.

Olde Loohuis, L. M. *et al.* (2018) 'Transcriptome analysis in whole blood reveals increased microbial diversity in schizophrenia', *Translational Psychiatry*. Nature Publishing Group, 8(1), p. 96. doi: 10.1038/s41398-018-0107-9.

Olsen-Bergem, H. *et al.* (2016) 'Juvenile idiopathic arthritis and rheumatoid arthritis: Bacterial diversity in temporomandibular joint synovial fluid in comparison with immunological and clinical findings', *International Journal of Oral and Maxillofacial Surgery*. doi: 10.1016/j.ijom.2015.08.986.

Ooi, K. G. J. *et al.* (2006) 'Cytokines and chemokines in uveitis - Is there a correlation with clinical phenotype?', *Clinical Medicine and Research*. doi: 10.3121/cmr.4.4.294.

Ortiz, P. *et al.* (2009) 'Periodontal Therapy Reduces the Severity of Active Rheumatoid Arthritis in Patients Treated With or Without Tumor Necrosis Factor Inhibitors', *Journal of Periodontology*. John Wiley & Sons, Ltd, 80(4), pp. 535–540. doi: 10.1902/jop.2009.080447.

de Pablo, P., Dietrich, T. and McAlindon, T. E. (2008) 'Association of periodontal disease

and tooth loss with rheumatoid arthritis in the US population.', *The Journal of rheumatology*. The Journal of Rheumatology, 35(1), pp. 70–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18050377.

Païssé, S. *et al.* (2016) 'Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing', *Transfusion*. John Wiley & Sons, Ltd (10.1111), 56(5), pp. 1138–1147. doi: 10.1111/trf.13477.

Palm, N. W. *et al.* (2014) 'Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease', *Cell*. doi: 10.1016/j.cell.2014.08.006.

PALMER, J. L. and BERTONE, A. L. (1994) 'Experimentally-induced synovitis as a model for acute synovitis in the horse', *Equine Veterinary Journal*, 26(6), pp. 492–495. doi: 10.1111/j.2042-3306.1994.tb04056.x.

Pan, F. *et al.* (2017) 'Dendritic Cells from Rheumatoid Arthritis Patient Peripheral Blood Induce Th17 Cell Differentiation via miR-363/Integrin *α* v/TGF- *β* Axis', *Scandinavian Journal of Immunology*, 85(6), pp. 441–449. doi: 10.1111/sji.12550.

Panaiotov, S., Filevski, G., Equestre, M., Nikolova, E., Kalfin, R., Panaiotov, S., *et al.* (2018) 'Cultural Isolation and Characteristics of the Blood Microbiome of Healthy Individuals', *Advances in Microbiology*. Scientific Research Publishing, 08(05), pp. 406–421. doi: 10.4236/aim.2018.85027.

Panaiotov, Stefan, Filevski, G., Equestre, M., Nikolova, E., Kalfin, R. and Panaiotov, S (2018) 'Cultural Isolation and Characteristics of the Blood Microbiome of Healthy Individuals', *Advances in Microbiology*, 8, pp. 406–421. doi: 10.4236/aim.2018.85027.

Paramsothy, S. *et al.* (2017) 'Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial', *The Lancet*. doi: 10.1016/S0140-6736(17)30182-4.

Park, S. *et al.* (2000) 'Rapid Identification of Candida dubliniensis Using a Species-Specific Molecular Beacon', *Journal of Clinical Microbiology*. American Society for Microbiology Journals, 38(8), pp. 2829–2836. Available at: https://jcm.asm.org/content/38/8/2829.short.

Peljto, A. L. *et al.* (2013) 'Association between the MUC5B promoter polymorphism and survival in patients with idiopathic pulmonary fibrosis', *JAMA - Journal of the American Medical Association*. doi: 10.1001/jama.2013.5827.

Penttinen, M. A. *et al.* (2004) 'Enhanced intracellular replication of Salmonella enteritidis in HLA-B27-expressing human monocytic cells: Dependency on glutamic acid at position 45 in the B pocket of HLA-B27', *Arthritis and Rheumatism*. doi: 10.1002/art.20336.

Peppercorn, M. A. and Goldman, P. (1972) 'The role of intestinal bacteria in the metabolism of salicylazosulfapyridine.', *Journal of Pharmacology and Experimental Therapeutics*.

Pessi, T. *et al.* (2015) 'Interleukin-6 and microRNA profiles induced by oral bacteria in human atheroma derived and healthy smooth muscle cells', *SpringerPlus*. SpringerOpen, 4(1), p. 206. doi: 10.1186/s40064-015-0993-8.

Peters, B. A. *et al.* (2017a) 'The oral fungal mycobiome: characteristics and relation to

periodontitis in a pilot study', *BMC Microbiology*, 17(1), p. 157. doi: 10.1186/s12866-017-1064-9.

Peters, B. A. *et al.* (2017b) 'The oral fungal mycobiome: characteristics and relation to periodontitis in a pilot study', *BMC Microbiology*, 17(1), p. 157. doi: 10.1186/s12866-017-1064-9.

Peters, B. A. *et al.* (2018) 'A taxonomic signature of obesity in a large study of American adults', *Scientific Reports*. doi: 10.1038/s41598-018-28126-1.

Peters, D. M. *et al.* (2014) 'TGF-β directs trafficking of the epithelial sodium channel ENaC which has implications for ion and fluid transport in acute lung injury', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(3), pp. E374–E383. doi: 10.1073/pnas.1306798111.

Peterson, D. A. *et al.* (2007) 'IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis', *Cell Host and Microbe*. doi: 10.1016/j.chom.2007.09.013.

Picchianti-Diamanti, A. *et al.* (2018) 'Analysis of Gut Microbiota in Rheumatoid Arthritis Patients: Disease-Related Dysbiosis and Modifications Induced by Etanercept', *International Journal of Molecular Sciences*, 19(10), p. 2938. doi: 10.3390/ijms19102938.

Podojil, J. R. and Miller, S. D. (2009) 'Molecular mechanisms of T-cell receptor and costimulatory molecule ligation/blockade in autoimmune disease therapy', *Immunological Reviews*, pp. 337–355. doi: 10.1111/j.1600-065X.2009.00773.x.

Potgieter, M. *et al.* (2015) 'The dormant blood microbiome in chronic, inflammatory diseases', *FEMS Microbiology Reviews*. Edited by P. A. Danchin. Oxford University Press, 39(4), pp. 567–591. doi: 10.1093/femsre/fuv013.

Pretorius, E. *et al.* (2017) 'Major involvement of bacterial components in rheumatoid arthritis and its accompanying oxidative stress, systemic inflammation and hypercoagulability', *Experimental Biology and Medicine*. doi: 10.1177/1535370216681549.

Przepiera-Będzak, H., Fischer, K. and Brzosko, M. (2015) 'Serum IL-6 and IL-23 Levels and Their Correlation with Angiogenic Cytokines and Disease Activity in Ankylosing Spondylitis, Psoriatic Arthritis, and SAPHO Syndrome', *Mediators of Inflammation*. Hindawi, 2015, pp. 1–7. doi: 10.1155/2015/785705.

Pull, S. L. *et al.* (2005) 'Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(1), pp. 99–104. doi: 10.1073/pnas.0405979102.

Qi, C.-J. *et al.* (2016) 'Imbalance of Fecal Microbiota at Newly Diagnosed Type 1 Diabetes in Chinese Children.', *Chinese medical journal*. Wolters Kluwer Health, 129(11), pp. 1298–304. doi: 10.4103/0366-6999.182841.

Qin, J. *et al.* (2010) 'A human gut microbial gene catalogue established by metagenomic sequencing', *Nature*, 464(7285), pp. 59–65. doi: 10.1038/nature08821.

Qin, J. *et al.* (2012) 'A metagenome-wide association study of gut microbiota in type 2 diabetes', *Nature*, 490(7418), pp. 55–60. doi: 10.1038/nature11450.

Rajaei, E. *et al.* (2019) 'Evaluating the relationship between serum level of interleukin-6 and rheumatoid arthritis severity and disease activity.', *Current rheumatology reviews*. doi: 10.2174/1573397115666190206144223.

Rajappan, S., Joseph, R. and Paul, B. J. (2015) *Relationship between Generalized Aggressive Periodontitis and Rheumatoid Arthritis: A Case Control Study*, *International Journal of Science and Research (IJSR) ISSN*.

Ramnani, P. *et al.* (2012) 'Invitro fermentation and prebiotic potential of novel low molecular weight polysaccharides derived from agar and alginate seaweeds', *Anaerobe*. doi: 10.1016/j.anaerobe.2011.08.003.

Rao, D. A. *et al.* (2017) 'Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis', *Nature*. doi: 10.1038/nature20810.

Rao, D. A. (2018) 'T cells that help B cells in chronically inflamed tissues', *Frontiers in Immunology*. Frontiers Media S.A. doi: 10.3389/fimmu.2018.01924.

Ravel, J. *et al.* (2011) 'Vaginal microbiome of reproductive-age women', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 108(SUPPL. 1), pp. 4680–4687. doi: 10.1073/pnas.1002611107.

Reboldi, A. *et al.* (2016) 'Mucosal immunology: IgA production requires B cell interaction with subepithelial dendritic cells in Peyer's patches', *Science*. doi: 10.1126/science.aaf4822.

Rehaume, L. *et al.* (2017) 'IL-23 promotes fecal microbiota dysbiosis associated with susceptibility to spondyloarthritis and ileitis in ZAP-70 mutant SKG mice', *espace.library.uq.edu.au*. Available at: https://espace.library.uq.edu.au/view/UQ:692191.

Reichardt, N. *et al.* (2014) 'Phylogenetic distribution of three pathways for propionate production within the human gut microbiota', *ISME Journal*. doi: 10.1038/ismej.2014.14.

Reichert, S. *et al.* (2013) 'Detection of oral bacterial DNA in synovial fluid', *Journal of Clinical Periodontology*. John Wiley & Sons, Ltd (10.1111), 40(6), pp. 591–598. doi: 10.1111/jcpe.12102.

Reid, G. *et al.* (2011) 'Microbiota restoration: natural and supplemented recovery of human microbial communities', *Nature Reviews Microbiology*. Nature Publishing Group, 9(1), pp. 27–38. doi: 10.1038/nrmicro2473.

Relman, D. A. (1999) 'The search for unrecognized pathogens.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 284(5418), pp. 1308–10. doi: 10.1126/SCIENCE.284.5418.1308.

Relman, D. A. and Falkow, S. (1992) 'Identification of uncultured microorganisms: expanding the spectrum of characterized microbial pathogens.', *Infectious agents and disease*, 1(5), pp. 245–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1285351.

Relman, D. A. and Falkow, S. (2001) 'The meaning and impact of the human genome sequence for microbiology.', *Trends in microbiology*. Elsevier, 9(5), pp. 206–8. doi: 10.1016/S0966-842X(01)02041-8.

Rescigno, M. (2011) 'Dendritic cells in bacteria handling in the gut', *J. Leukoc. Biol*, 90, pp.

669–672. doi: 10.1189/jlb.0311141.

Riedhammer, C. and Weissert, R. (2015) 'Antigen presentation, autoantigens, and immune regulation in multiple sclerosis and other autoimmune diseases', *Frontiers in Immunology*. Frontiers Media S.A. doi: 10.3389/fimmu.2015.00322.

Rizkallah, M., Saad, R. and Aziz, R. (2012) 'The Human Microbiome Project, Personalized Medicine and the Birth of Pharmacomicrobiomics', *Current Pharmacogenomics and Personalized Medicine*. doi: 10.2174/187569210792246326.

Roager, H. M. and Licht, T. R. (2018) 'Microbial tryptophan catabolites in health and disease', *Nature Communications*. doi: 10.1038/s41467-018-05470-4.

Roark, C. L. *et al.* (2007) 'Exacerbation of Collagen-Induced Arthritis by Oligoclonal, IL-17-Producing T Cells', *The Journal of Immunology*. doi: 10.4049/jimmunol.179.8.5576.

da Rocha, L. F. *et al.* (2012a) 'Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity.', *The Journal of rheumatology*. The Journal of Rheumatology, 39(7), pp. 1320–5. doi: 10.3899/jrheum.111027.

da Rocha, L. F. *et al.* (2012b) 'Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity.', *The Journal of rheumatology*. The Journal of Rheumatology, 39(7), pp. 1320–5. doi: 10.3899/jrheum.111027.

Rogier, R. *et al.* (2017) 'Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis', *Microbiome*. doi: 10.1186/s40168-017-0278-2.

Rojo, D. *et al.* (2017) 'Exploring the human microbiome from multiple perspectives: factors altering its composition and function', *FEMS Microbiology Reviews*, 41(4), pp. 453–478. doi: 10.1093/femsre/fuw046.

Rosenbaum, J. T. and Davey, M. P. (2011) 'Time for a gut check: Evidence for the hypothesis that HLA-B27 predisposes to ankylosing spondylitis by altering the microbiome', *Arthritis and Rheumatism*. John Wiley & Sons, Ltd, pp. 3195–3198. doi: 10.1002/art.30558.

Ross, J. J. (2017) 'Septic Arthritis of Native Joints.', *Infectious disease clinics of North America*. Elsevier, 31(2), pp. 203–218. doi: 10.1016/j.idc.2017.01.001.

Rossol, M. *et al.* (2011) 'LPS-induced cytokine production in human monocytes and macrophages', *Critical Reviews in Immunology*.

Roşu, A. *et al.* (2012) 'IL-17 patterns in synovium, serum and synovial fluid from treatment-naïve, early rheumatoid arthritis patients.', *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie*, 53(1), pp. 73–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22395503.

Round, J. L. and Mazmanian, S. K. (2010) 'Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(27), pp. 12204–9. doi: 10.1073/pnas.0909122107.

Roux, S. Le *et al.* (2016) 'Transforming Growth Factor Beta 1 Modulates the Functional Expression of the Neurokinin-1 Receptor in Human Keratocytes', *Current Eye Research*.

doi: 10.3109/02713683.2015.1088954.

Ruiz-Ruiz, C. *et al.* (2011) 'An exopolysaccharide produced by the novel halophilic bacterium Halomonas stenophila strain B100 selectively induces apoptosis in human T leukaemia cells', *Applied Microbiology and Biotechnology*. doi: 10.1007/s00253-010-2886-7.

Ruuska, M. *et al.* (2013) 'Phosphorylation of STAT-1 Serine 727 Is Prolonged in HLA-B27-Expressing Human Monocytic Cells', *PLoS ONE*. doi: 10.1371/journal.pone.0050684.

Sack, U. *et al.* (1993) 'Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis.', *Rheumatology international*, 13(2), pp. 45–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8356390

Sagar, S. *et al.* (2013) 'Induction of apoptosis in cancer cell lines by the Red Sea brine pool bacterial extracts', *BMC Complementary and Alternative Medicine*. doi: 10.1186/1472-6882-13-344.

Sahlberg, A. S. *et al.* (2007) 'Evidence that the p38 MAP kinase pathway is dysregulated in HLA-B27-expressing human monocytic cells: Correlation with HLA-B27 misfolding', *Arthritis and Rheumatism*. doi: 10.1002/art.22746.

Sakamoto, K. *et al.* (2012) 'Acute exacerbation of IPF following diagnostic bronchoalveolar lavage procedures', *Respiratory Medicine*. doi: 10.1016/j.rmed.2011.11.006.

Salmon-Ceron, D. *et al.* (2011) 'Drug-specific risk of non-tuberculosis opportunistic infections in patients receiving anti-TNF therapy reported to the 3-year prospective French RATIO registry.', *Annals of the rheumatic diseases*. BMJ Publishing Group Ltd, 70(4), pp. 616–23. doi: 10.1136/ard.2010.137422.

Salter, S. J. *et al.* (2014a) 'Reagent and laboratory contamination can critically impact sequence-based microbiome analyses', *BMC Biology*. BioMed Central, 12(1), p. 87. doi: 10.1186/s12915-014-0087-z.

Salter, S. J. *et al.* (2014b) *Reagent and laboratory contamination can critically impact sequence-based microbiome analyses*. doi: 10.1186/s12915-014-0087-z.

Sandoval-Denis, M. *et al.* (2015) 'Cladosporium Species Recovered from Clinical Samples in the United States', *Journal of Clinical Microbiology*. Edited by D. W. Warnock, 53(9), pp. 2990–3000. doi: 10.1128/JCM.01482-15.

Santiago, A. *et al.* (2016) 'Alteration of the serum microbiome composition in cirrhotic patients with ascites', *Scientific Reports*. Nature Publishing Group, 6(1), p. 25001. doi: 10.1038/srep25001.

Sarkar, S. (2005) 'Dendritic cells in rheumatoid arthritis NET ins Rheumatoid Arthritis View project Roles for Th17 cells in autoimmunity View project', pp. 656–665. doi: 10.2741/1560.

Sarkar, S. and Fox, D. A. (2005) 'Dendritic cells in rheumatoid arthritis.', *Frontiers in bioscience : a journal and virtual library*, pp. 656–665. doi: 10.2741/1560.

Sato, K. *et al.* (2006) 'Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction.', *The Journal of experimental medicine*. doi: 10.1084/jem.20061775.

Scannapieco, F. A., Bush, R. B. and Paju, S. (2003) 'Associations Between Periodontal Disease and Risk for Atherosclerosis, Cardiovascular Disease, and Stroke. A Systematic Review', *Annals of Periodontology*, 8(1), pp. 38–53. doi: 10.1902/annals.2003.8.1.38.

Scharf, V. *et al.* (2015) 'Comparison of synovial fluid culture and 16S rRNA PCR in dogs with suspected septic arthritis', *Australian Veterinary Journal*, 93(6), pp. 204–207. doi: 10.1111/avj.12329.

Schellekens, G. A. *et al.* (1998) 'Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies', *Journal of Clinical Investigation*. doi: 10.1172/JCI1316.

Schellekens, G. A. *et al.* (2000) 'The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide', *Arthritis and Rheumatism*. doi: 10.1002/1529-0131(200001)43:1<155::AID-ANR20>3.0.CO;2-3.

Scher, J. U. *et al.* (2012) 'Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis', *Arthritis & Rheumatism*. John Wiley & Sons, Ltd, 64(10), pp. 3083–3094. doi: 10.1002/art.34539.

Scher, J. U. *et al.* (2013) 'Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis', 2, p. 1202. doi: 10.7554/eLife.01202.

Scher, J. U. *et al.* (2015) 'Decreased Bacterial Diversity Characterizes the Altered Gut Microbiota in Patients With Psoriatic Arthritis, Resembling Dysbiosis in Inflammatory Bowel Disease', *Arthritis & Rheumatology*, 67(1), pp. 128–139. doi: 10.1002/art.38892.

Scher, J. U. *et al.* (2016) 'The lung microbiota in early rheumatoid arthritis and autoimmunity', *Microbiome*. BioMed Central, 4(1), p. 60. doi: 10.1186/s40168-016-0206-x.

Scher, J. U. *et al.* (2020) 'Pharmacomicrobiomics in inflammatory arthritis: gut microbiome as modulator of therapeutic response', *Nature Reviews Rheumatology*. doi: 10.1038/s41584-020-0395-3.

Scher, J. U. and Abramson, S. B. (2011) 'The microbiome and rheumatoid arthritis', *Nature Reviews Rheumatology*. Nature Publishing Group, 7(10), pp. 569–578. doi: 10.1038/nrrheum.2011.121.

Schlegel, P. M. *et al.* (2013) 'B cells contribute to heterogeneity of IL-17 producing cells in rheumatoid arthritis and healthy controls', *PLoS ONE*, 8(12). doi: 10.1371/journal.pone.0082580.

Schreiber, F., Arasteh, J. M. and Lawley, T. D. (2015) 'Pathogen Resistance Mediated by IL-22 Signaling at the Epithelial–Microbiota Interface', *Journal of Molecular Biology*. Academic Press, 427(23), pp. 3676–3682. doi: 10.1016/J.JMB.2015.10.013.

Schroeder, B. O. and Bäckhed, F. (2016) 'Signals from the gut microbiota to distant organs in physiology and disease', *Nature Medicine*. doi: 10.1038/nm.4185.

Schuijt, T. J. *et al.* (2013) 'The intestinal microbiota and host immune interactions in the critically ill', *Trends in Microbiology*. Elsevier Current Trends, 21(5), pp. 221–229. doi: 10.1016/J.TIM.2013.02.001.

Schulze-Koops, H. and Kalden, J. R. (2001) 'The balance of Th1/Th2 cytokines in

rheumatoid arthritis', *Best Practice and Research: Clinical Rheumatology*. Bailliere Tindall Ltd, 15(5), pp. 677–691. doi: 10.1053/berh.2001.0187.

Sebbag, M. *et al.* (1995) 'The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies', *Journal of Clinical Investigation*. doi: 10.1172/JCI117969.

Seck, E. h. *et al.* (2016) '"Halomonas massiliensis" sp. nov., a new halotolerant bacterium isolated from the human gut', *New Microbes and New Infections*, 14, pp. 19–20. doi: 10.1016/j.nmni.2016.07.013.

Segal, L. N. *et al.* (2013) 'Enrichment of lung microbiome with supraglottic taxa is associated with increased pulmonary inflammation', *Microbiome*. BioMed Central, 1(1), p. 19. doi: 10.1186/2049-2618-1-19.

Sękowska, A. (2017) 'Raoultella spp.—clinical significance, infections and susceptibility to antibiotics', *Folia Microbiologica*, 62(3), pp. 221–227. doi: 10.1007/s12223-016-0490-7.

Sender, R., Fuchs, S. and Milo, R. (2016) 'Revised Estimates for the Number of Human and Bacteria Cells in the Body', *PLOS Biology*. Public Library of Science, 14(8), p. e1002533. doi: 10.1371/journal.pbio.1002533.

Seng, P. *et al.* (2016) 'Emerging role of Raoultella ornithinolytica in human infections: a series of cases and review of the literature', *International Journal of Infectious Diseases*, 45, pp. 65–71. doi: 10.1016/j.ijid.2016.02.014.

Shchipkova, A. Y., Nagaraja, H. N. and Kumar, P. S. (2010) 'Subgingival microbial profiles of smokers with periodontitis', *Journal of Dental Research*. doi: 10.1177/0022034510377203.

Shen, H., Rogelj, S. and Kieft, T. L. (2006) 'Sensitive, real-time PCR detects low-levels of contamination by Legionella pneumophila in commercial reagents', *Molecular and Cellular Probes*. Academic Press, 20(3–4), pp. 147–153. doi: 10.1016/J.MCP.2005.09.007.

Shi, Y. *et al.* (2016) 'Fecal microbiota transplantation for ulcerative colitis: A systematic review and meta-analysis', *PLoS ONE*. doi: 10.1371/journal.pone.0157259.

Shibata, K. *et al.* (2007) 'Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production.', *Journal of Immunology*. doi: 178/7/4466 [pii].

Shin, J. H. *et al.* (2016) 'Lifestyle and geographic insights into the distinct gut microbiota in elderly women from two different geographic locations', *Journal of Physiological Anthropology*. doi: 10.1186/s40101-016-0121-7.

Shmerling, R. H. and Delbanco, T. L. (1991) 'The rheumatoid factor: an analysis of clinical utility.', *The American journal of medicine*, 91(5), pp. 528–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1951415.

Siala, M. *et al.* (2008) 'Analysis of bacterial DNA in synovial tissue of Tunisian patients with reactive and undifferentiated arthritis by broad-range PCR, cloning and sequencing', *Arthritis Research & Therapy*, 10(2), p. R40. doi: 10.1186/ar2398.

Silverman, G. J. and Carson, D. A. (2003) 'Roles of B cells in rheumatoid arthritis', *Arthritis*

Research & Therapy, 5(Suppl 4), p. S1. doi: 10.1186/ar1010.

Singh, H. *et al.* (2003) 'Pulmonary Aspergilloma in Rheumatoid Arthritis.', *Medical journal, Armed Forces India*. Elsevier, 59(3), pp. 254–6. doi: 10.1016/S0377-1237(03)80024-2.

Singh, P., Hashmi, G. and Swain, P. K. (2018) 'High prevalence of cesarean section births in private sector health facilities- analysis of district level household survey-4 (DLHS-4) of India', *BMC Public Health*, 18(1), p. 613. doi: 10.1186/s12889-018-5533-3.

Singh, P. and Manning, S. D. (2016) 'Impact of age and sex on the composition and abundance of the intestinal microbiota in individuals with and without enteric infections', *Annals of Epidemiology*. doi: 10.1016/j.annepidem.2016.03.007.

Singh, R. K. *et al.* (2017) 'Influence of diet on the gut microbiome and implications for human health', *Journal of Translational Medicine*. BioMed Central Ltd. doi: 10.1186/s12967-017-1175-y.

Siouti, E. and Andreakos, E. (2019) 'The many facets of macrophages in rheumatoid arthritis', *Biochemical Pharmacology*. Elsevier Inc., pp. 152–169. doi: 10.1016/j.bcp.2019.03.029.

Sköldstam, L., Hagfors, L. and Johansson, G. (2003) 'An experimental study of a Mediterranean diet intervention for patients with rheumatoid arthritis', *Annals of the Rheumatic Diseases*. doi: 10.1136/ard.62.3.208.

Smid, M. *et al.* (2018) 'Maternal Gut Microbiome Biodiversity in Pregnancy', *American Journal of Perinatology*, 35(01), pp. 024–030. doi: 10.1055/s-0037-1604412.

Smith, P. M. *et al.* (2013) 'The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic Treg Cell Homeostasis', *Science*, 341(6145), pp. 569–573. doi: 10.1126/science.1241165.

Smits, L. P. *et al.* (2013) 'Therapeutic potential of fecal microbiota transplantation', *Gastroenterology*. doi: 10.1053/j.gastro.2013.08.058.

Smolen, J. S. *et al.* (2007) 'New therapies for treatment of rheumatoid arthritis', *The Lancet*. Elsevier, 370(9602), pp. 1861–1874. doi: 10.1016/S0140-6736(07)60784-3.

Smolen, J. S. *et al.* (2014) 'EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update', *Annals of the Rheumatic Diseases*, 73(3), pp. 492–509. doi: 10.1136/annrheumdis-2013-204573.

Smolen, J. S. *et al.* (2020) 'EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update', *Ann Rheum Dis*, 0, pp. 1–15. doi: 10.1136/annrheumdis-2019-216655.

Sokol, H. *et al.* (2017) 'Fungal microbiota dysbiosis in IBD', *Gut*, 66(6), pp. 1039–1048. doi: 10.1136/gutjnl-2015-310746.

Sommer, O. J. *et al.* (2005) 'Rheumatoid Arthritis: A Practical Guide to State-of-the-Art Imaging, Image Interpretation, and Clinical Implications', *RadioGraphics*. Radiological Society of North America , 25(2), pp. 381–398. doi: 10.1148/rg.252045111.

Song, W. *et al.* (1994) 'Stimulation of transcytosis of the polymeric immunoglobulin receptor by dimeric IgA', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.91.1.163.

Sonnenberg, G. F., Fouser, L. A. and Artis, D. (2011) 'Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22', *Nature Immunology*. Nature Publishing Group, 12(5), pp. 383–390. doi: 10.1038/ni.2025.

Soós, L. *et al.* (2007) 'Clinical evaluation of anti-mutated citrullinated vimentin by ELISA in rheumatoid arthritis.', *The Journal of rheumatology*, 34(8), pp. 1658–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17611988.

SOX, H. C. and LIANG, M. H. (1986) 'Diagnostic Decision: The Erythrocyte Sedimentation Rate: Guidelines for Rational Use', *Annals of Internal Medicine*. American College of Physicians, 104(4), p. 515. doi: 10.7326/0003-4819-104-4-515.

Sparks, J. A. *et al.* (2018) 'Weight Change During the Early Rheumatoid Arthritis Period and Risk of Subsequent Mortality in Women With Rheumatoid Arthritis and Matched Comparators', *Arthritis and Rheumatology*. doi: 10.1002/art.40346.

Srirangan, S. and Choy, D. E. H. (2010) 'The Role of Interleukin 6 in the Pathophysiology of Rheumatoid Arthritis', *Therapeutic Advances in Musculoskeletal Disease*. SAGE Publications, 2(5), p. 247. doi: 10.1177/1759720X10378372.

Srirangan, S. and Choy, E. H. (2010) 'The role of Interleukin 6 in the pathophysiology of rheumatoid arthritis', *Therapeutic Advances in Musculoskeletal Disease*. SAGE PublicationsSage UK: London, England, 2(5), pp. 247–256. doi: 10.1177/1759720X10378372.

Stepankova, R. *et al.* (2007) 'Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells', *Inflammatory Bowel Diseases*. Oxford University Press, 13(10), pp. 1202–1211. doi: 10.1002/ibd.20221.

Stinson, L. F., Keelan, J. A. and Payne, M. S. (2019) 'Identification and removal of contaminating microbial DNA from PCR reagents: impact on low-biomass microbiome analyses', *Letters in Applied Microbiology*. John Wiley & Sons, Ltd (10.1111), 68(1), pp. 2–8. doi: 10.1111/lam.13091.

Stinson, L. F., Payne, M. S. and Keelan, J. A. (2018) 'A Critical Review of the Bacterial Baptism Hypothesis and the Impact of Cesarean Delivery on the Infant Microbiome', *Frontiers in Medicine*. Frontiers, 5, p. 135. doi: 10.3389/fmed.2018.00135.

Stojanovich, L. and Marisavljevich, D. (2008) 'Stress as a trigger of autoimmune disease', *Autoimmunity Reviews*. doi: 10.1016/j.autrev.2007.11.007.

Strand, V., Kimberly, R. and Isaacs, J. D. (2007) 'Biologic therapies in rheumatology: Lessons learned, future directions', *Nature Reviews Drug Discovery*, pp. 75–92. doi: 10.1038/nrd2196.

Straub, R. *et al.* (1997) 'Decrease of interleukin 6 during the first 12 months is a prognostic marker for clinical outcome during 36 months treatment with disease- modifying anti-rheumatic drugs', *Rheumatology*. Oxford University Press, 36(12), pp. 1298–1303. doi: 10.1093/rheumatology/36.12.1298.

Sun, D. *et al.* (2016) 'Fecal Microbiota Transplantation as a Novel Therapy for Ulcerative Colitis', *Medicine*. doi: 10.1097/md.0000000000003765.

Sun, Y. *et al.* (2019) 'Characteristics of Gut Microbiota in Patients With Rheumatoid Arthritis in Shanghai, China', *Frontiers in Cellular and Infection Microbiology*. Frontiers Media S.A., 9, p. 369. doi: 10.3389/fcimb.2019.00369.

Surawicz, C. M. *et al.* (2013) 'Guidelines for diagnosis, treatment, and prevention of clostridium difficile infections', *American Journal of Gastroenterology*. doi: 10.1038/ajg.2013.4.

Suzuki, A. *et al.* (2003) 'Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis', *Nature Genetics*, 34(4), pp. 395–402. doi: 10.1038/ng1206.

Suzuki, K. *et al.* (2007) 'Intestinal IgA synthesis: A primitive form of adaptive immunity that regulates microbial communities in the gut', *Seminars in Immunology*. Academic Press, 19(2), pp. 127–135. doi: 10.1016/J.SMIM.2006.10.001.

Sverrild, A. *et al.* (2017) 'Eosinophilic airway inflammation in asthmatic patients is associated with an altered airway microbiome', *Journal of Allergy and Clinical Immunology*, 140(2), pp. 407-417.e11. doi: 10.1016/j.jaci.2016.10.046.

Swaak, A. J. *et al.* (1988) 'Interleukin-6 (IL-6) in Synovial Fluid and Serum of Patients with Rheumatic Diseases', *Scandinavian Journal of Rheumatology*. Taylor & Francis, 17(6), pp. 469–474. doi: 10.3109/03009748809098809.

Szilagyi, A. (2020) 'Relationship(s) between obesity and inflammatory bowel diseases: possible intertwined pathogenic mechanisms', *Clinical Journal of Gastroenterology*. doi: 10.1007/s12328-019-01037-y.

Takatori, H. *et al.* (2008) 'Lymphoid tissue inducer–like cells are an innate source of IL-17 and IL-22', *The Journal of experimental medicine*. doi: 10.1084/jem.20072713.

Talham, G. L. *et al.* (1999) 'Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system', *Infection and Immunity*.

Tanaka, S. *et al.* (2013) 'Lipopolysaccharide accelerates collagen-induced arthritis in association with rapid and continuous production of inflammatory mediators and anti-type II collagen antibody', *Microbiology and Immunology*, 57(6), pp. 445–454. doi: 10.1111/1348-0421.12052.

Taneja, V. (2014) 'Arthritis susceptibility and the gut microbiome', *FEBS Letters*, 588(22), pp. 4244–4249. doi: 10.1016/j.febslet.2014.05.034.

Tatro, E. T. *et al.* (2014) 'Antibody Response to Achromobacter xylosoxidans during HIV Infection Is Associated with Lower CD4 Levels and Increased Lymphocyte Activation', *Clinical and Vaccine Immunology*. Edited by V. M. Litwin, 21(1), pp. 46–50. doi: 10.1128/CVI.00553-13.

du Teil Espina, M. *et al.* (2019) 'Talk to your gut: the oral-gut microbiome axis and its immunomodulatory role in the etiology of rheumatoid arthritis.', *FEMS microbiology reviews*, 43(1), pp. 1–18. doi: 10.1093/femsre/fuy035.

Témoin, S. *et al.* (2012a) 'Identification of oral bacterial DNA in synovial fluid of patients with arthritis with native and failed prosthetic joints.', *Journal of clinical rheumatology : practical reports on rheumatic & musculoskeletal diseases*. NIH Public Access, 18(3), pp. 117–21. doi: 10.1097/RHU.0b013e3182500c95.

Témoin, S. *et al.* (2012b) 'Identification of oral bacterial DNA in synovial fluid of patients with arthritis with native and failed prosthetic joints.', *Journal of clinical rheumatology : practical reports on rheumatic & musculoskeletal diseases*. NIH Public Access, 18(3), pp. 117–21. doi: 10.1097/RHU.0b013e3182500c95.

Teng, F. *et al.* (2016) 'Gut Microbiota Drive Autoimmune Arthritis by Promoting Differentiation and Migration of Peyer's Patch T Follicular Helper Cells', *Immunity*. Cell Press, 44(4), pp. 875–888. doi: 10.1016/j.immuni.2016.03.013.

Tirado-Rodriguez, B. *et al.* (2014) 'TGF-β: An important mediator of allergic disease and a molecule with dual activity in cancer development', *Journal of Immunology Research*. doi: 10.1155/2014/318481.

Tortora, G., Case, C. and Funke, B. (2016) *Microbiologia-12ª Edição*. Available at: https://books.google.co.uk/books?hl=en&lr=&id=L98_DQAAQBAJ&oi=fnd&pg=PR1&dq=-+Tortora+GJ,+Case+CL,+Funke+BR.+Microbiologia-12ª+Edição.+Artmed+Editora%3B+2016+Oct+1&ots=E2OxjqzzTv&sig=10ToU5ZkDTz1TdW39I1bw7FeMJM.

Travis, S. M. *et al.* (1999) 'Activity of abundant antimicrobials of the human airway', *American Journal of Respiratory Cell and Molecular Biology*. doi: 10.1165/ajrcmb.20.5.3572.

Treiner, E. *et al.* (2003) 'Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1', *Nature*. doi: 10.1038/nature01433.

Trøseid, M. *et al.* (2014) 'Microbial Translocation and Cardiometabolic Risk Factors in HIV Infection', *AIDS Research and Human Retroviruses*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA , 30(6), pp. 514–522. doi: 10.1089/aid.2013.0280.

Tsai, M.-S. *et al.* (2008) 'Shewanella soft tissue infection: case report and literature review', *International Journal of Infectious Diseases*, 12(6), pp. e119–e124. doi: 10.1016/j.ijid.2008.03.020.

Tsiodras, S. *et al.* (2008) 'Fungal Infections Complicating Tumor Necrosis Factor α Blockade Therapy', *Mayo Clinic Proceedings*. Elsevier, 83(2), pp. 181–194. doi: 10.4065/83.2.181.

Tsuji, M. *et al.* (2008) 'Requirement for Lymphoid Tissue-Inducer Cells in Isolated Follicle Formation and T Cell-Independent Immunoglobulin A Generation in the Gut', *Immunity*. doi: 10.1016/j.immuni.2008.05.014.

Turenne, C. Y. *et al.* (1999) *Rapid Identification of Fungi by Using the ITS2 Genetic Region and an Automated Fluorescent Capillary Electrophoresis System*, *JOURNAL OF CLINICAL MICROBIOLOGY*. Available at: http://jcm.asm.org/.

Turesson, C. *et al.* (2015) 'High serum cholesterol predicts rheumatoid arthritis in women, but not in men: a prospective study', *Arthritis Research & Therapy*. BioMed Central, 17(1),

p. 284. doi: 10.1186/s13075-015-0804-1.

Turnbaugh, P. J. *et al.* (2006) 'An obesity-associated gut microbiome with increased capacity for energy harvest', *Nature*. doi: 10.1038/nature05414.

Uematsu, H. *et al.* (2003) 'Degradation of arginine and other amino acids by butyrate-producing asaccharolytic anaerobic Gram-positive rods in periodontal pockets.', *Archives of oral biology*, 48(6), pp. 423–9. doi: 10.1016/S0003-9969(03)00031-1.

Urbaniak, C., Burton, J. P. and Reid, G. (2012) 'Breast, Milk and Microbes: A Complex Relationship that Does Not End with Lactation', *Women's Health*, 8(4), pp. 385–398. doi: 10.2217/WHE.12.23.

Utriainen, L. *et al.* (2012) 'Expression of HLA-B27 causes loss of migratory dendritic cells in a rat model of spondylarthritis', *Arthritis and Rheumatism*. doi: 10.1002/art.34561.

Uyeno, Y., Sekiguchi, Y. and Kamagata, Y. (2008) 'Impact of consumption of probiotic lactobacilli-containing yogurt on microbial composition in human feces', *International Journal of Food Microbiology*. doi: 10.1016/j.ijfoodmicro.2007.11.042.

Vaahtovuo, J. *et al.* (2008) 'Fecal microbiota in early rheumatoid arthritis.', *The Journal of rheumatology*, 35(8), pp. 1500–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18528968.

Vaishnavi, C. (2013) 'Translocation of gut flora and its role in sepsis', *Indian Journal of Medical Microbiology*. doi: 10.4103/0255-0857.118870.

Vajro, P., Paolella, G. and Fasano, A. (2013) 'Microbiota and gut-liver axis: their influences on obesity and obesity-related liver disease.', *Journal of pediatric gastroenterology and nutrition*. NIH Public Access, 56(5), pp. 461–8. doi: 10.1097/MPG.0b013e318284abb5.

Valerino, D. M. *et al.* (1972) 'Studies of the metabolism of methotrexate by intestinal flora-I. Identification and study of biological properties of the metabolite 4-amino-4-deoxy-N10-methylpteroic acid', *Biochemical Pharmacology*. doi: 10.1016/0006-2952(72)90125-6.

Van Venrooij, W. J. and Zendman, A. J. W. (2008) 'Anti-CCP2 antibodies: An overview and perspective of the diagnostic abilities of this serological marker for early rheumatoid arthritis', *Clinical Reviews in Allergy and Immunology*. doi: 10.1007/s12016-007-8029-y.

Venus, K., Vaithilingam, S. and Bogoch, I. I. (2016) 'Septic arthritis of the knee due to Raoultella ornithinolytica', *Infection*, 44(5), pp. 691–692. doi: 10.1007/s15010-016-0930-y.

Verheul, M. K. *et al.* (2015) 'Biomarkers for rheumatoid and psoriatic arthritis', *Clinical Immunology*. doi: 10.1016/j.clim.2015.04.005.

Viladomiu, M. *et al.* (2017) 'IgA-coated E. Coli enriched in Crohn's disease spondyloarthritis promote TH17-dependent inflammation', *Science Translational Medicine*. doi: 10.1126/scitranslmed.aaf9655.

Villeneuve, E. *et al.* (2013) 'A systematic literature review of strategies promoting early referral and reducing delays in the diagnosis and management of inflammatory arthritis', *Annals of the Rheumatic Diseases*. doi: 10.1136/annrheumdis-2011-201063.

Villeneuve, E., Nam, J. and Emery, P. (2010) '2010 ACR-EULAR classification criteria for

rheumatoid arthritis.', *Revista brasileira de reumatologia*, 50(5), pp. 481–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21125184.

Vrieze, A. *et al.* (2012) 'Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome', *Gastroenterology*. doi: 10.1053/j.gastro.2012.06.031.

Wajid Ali Khan, M. and Ali Khan, W. (2019) 'Autoantibodies and Cytokines in Pathogenesis of Rheumatoid Arthritis', in *Autoantibodies and Cytokines*. IntechOpen. doi: 10.5772/intechopen.82265.

Waldram, A. *et al.* (2009) 'Top-Down Systems Biology Modeling of Host Metabotype−Microbiome Associations in Obese Rodents', *Journal of Proteome Research*, 8(5), pp. 2361–2375. doi: 10.1021/pr8009885.

Wallis, A. *et al.* (2016) 'Support for the Microgenderome: Associations in a Human Clinical Population', *Scientific Reports*. doi: 10.1038/srep19171.

Wang, E. A. *et al.* (2017) 'Targeting IL-17 in psoriatic arthritis.', *European journal of rheumatology*. AVES, 4(4), pp. 272–277. doi: 10.5152/eurjrheum.2017.17037.

Wang, H. X. and Wang, Y. P. (2016) 'Gut microbiota-brain axis', *Chinese Medical Journal*. doi: 10.4103/0366-6999.190667.

Wang, QuanQiu and Xu, R. (2019) 'Data-driven multiple-level analysis of gut-microbiome-immune-joint interactions in rheumatoid arthritis', *BMC Genomics*. BioMed Central, 20(1), p. 124. doi: 10.1186/s12864-019-5510-y.

Wang, Quanqiu and Xu, R. (2019) 'Data-driven multiple-level analysis of gut-microbiome-immune-joint interactions in rheumatoid arthritis', *BMC Genomics*. BioMed Central Ltd., 20(1). doi: 10.1186/s12864-019-5510-y.

Wang, X. J. *et al.* (2008) 'Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod Nassarius semiplicatus', *Toxicon*. doi: 10.1016/j.toxicon.2008.04.170.

Wang, Z. *et al.* (2011) 'Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease', *Nature*, 472(7341), pp. 57–63. doi: 10.1038/nature09922.

Wculek, S. K. *et al.* (2019) 'Dendritic cells in cancer immunology and immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group. doi: 10.1038/s41577-019-0210-z.

Wehkamp, J. *et al.* (2008) 'Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease', *Mucosal Immunology*. Nature Publishing Group, 1(1), pp. S67–S74. doi: 10.1038/mi.2008.48.

Wei, B. *et al.* (2008) 'Resident enteric microbiota and CD8+ T cells shape the abundance of marginal zone B cells', *European Journal of Immunology*. John Wiley & Sons, Ltd, 38(12), pp. 3411–3425. doi: 10.1002/eji.200838432.

Weiss, S. *et al.* (2014) 'Tracking down the sources of experimental contamination in microbiome studies', *Genome Biology*. BioMed Central, 15(12), p. 564. doi: 10.1186/s13059-014-0564-2.

Wendling, D. (2008) 'Interleukin 23: A key cytokine in chronic inflammatory disease', *Joint Bone Spine*, 75(5), pp. 517–519. doi: 10.1016/j.jbspin.2008.03.004.

Wesa, A. and Galy, A. (2002) 'Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand', *BMC Immunology*. BioMed Central, 3(1), p. 14. doi: 10.1186/1471-2172-3-14.

Weyrich, L. S. *et al.* (2019) 'Laboratory contamination over time during low-biomass sample analysis', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 19(4), pp. 1755–0998.13011. doi: 10.1111/1755-0998.13011.

Whittle, E. *et al.* (2019) 'Multi-Method Characterization of the Human Circulating Microbiome', *Frontiers in Microbiology*, 9, p. 3266. doi: 10.3389/fmicb.2018.03266.

Van De Wiele, T. *et al.* (2016) 'How the microbiota shapes rheumatic diseases', *Nature Reviews Rheumatology*. Nature Publishing Group, pp. 398–411. doi: 10.1038/nrrheum.2016.85.

Wiest, R., Lawson, M. and Geuking, M. (2014) 'Pathological bacterial translocation in liver cirrhosis.', *Journal of hepatology*. Elsevier, 60(1), pp. 197–209. doi: 10.1016/j.jhep.2013.07.044.

William, A. (2012) 'Blood Culture Systems: From Patient to Result', in *Sepsis - An Ongoing and Significant Challenge*. InTech. doi: 10.5772/50139.

Wilson, B. C. *et al.* (2019) 'The super-donor phenomenon in fecal microbiota transplantation', *Frontiers in Cellular and Infection Microbiology*. doi: 10.3389/fcimb.2019.00002.

Wingender, G. *et al.* (2012) 'Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice', *Gastroenterology*. doi: 10.1053/j.gastro.2012.04.017.

Witherden, E. A. *et al.* (2017) 'The Human Mucosal Mycobiome and Fungal Community Interactions', *Journal of Fungi*. Multidisciplinary Digital Publishing Institute (MDPI), 3(4), p. 56. doi: 10.3390/jof3040056.

Wittenbrink, M. M. (2012) 'Bacterial infections of the equine genital tract', *Pferdeheilkunde Equine Medicine*, 28(1), pp. 30–32. doi: 10.21836/PEM20120107.

Woese, C. R. (1987) *Bacterial Evolution*, *MICROBIOLOGICAL REVIEWS*. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC373105/pdf/microrev00049-0051.pdf.

Wolff, B. *et al.* (2017) 'FRI0698 Prevotella and alloprevotella species characterize the oral microbiome of early rheumatoid arthritis', in *Poster Presentations*. BMJ Publishing Group Ltd and European League Against Rheumatism, pp. 754.2-754. doi: 10.1136/annrheumdis-2017-eular.2874.

Wong, J. M. W. *et al.* (2006) 'Colonic health: fermentation and short chain fatty acids.', *Journal of clinical gastroenterology*, 40(3), pp. 235–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16633129.

Wu, H.-J. *et al.* (2010) 'Gut-Residing Segmented Filamentous Bacteria Drive Autoimmune Arthritis via T Helper 17 Cells', *Immunity*. Cell Press, 32(6), pp. 815–827. doi: 10.1016/J.IMMUNI.2010.06.001.

Wu, H.-J. and Wu, E. (2012) 'The role of gut microbiota in immune homeostasis and autoimmunity', *Gut Microbes*. Taylor & Francis, 3(1), pp. 4–14. doi: 10.4161/gmic.19320.

Wu, M. *et al.* (2020) 'Antibiotic-induced dysbiosis of gut microbiota impairs corneal development in postnatal mice by affecting CCR2 negative macrophage distribution', *Mucosal Immunology*. doi: 10.1038/s41385-019-0193-x.

Wu, P. *et al.* (2018) 'Profiling the Urinary Microbiota in Male Patients With Bladder Cancer in China.', *Frontiers in cellular and infection microbiology*. Frontiers Media SA, 8, p. 167. doi: 10.3389/fcimb.2018.00167.

Wu, W. *et al.* (2016) 'Commensal A4 bacteria inhibit intestinal Th2-cell responses through induction of dendritic cell TGF-β production', *European Journal of Immunology*. John Wiley & Sons, Ltd, 46(5), pp. 1162–1167. doi: 10.1002/eji.201546160.

Wu, X. *et al.* (2016) 'Molecular Insight into Gut Microbiota and Rheumatoid Arthritis', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute, 17(3), p. 431. doi: 10.3390/ijms17030431.

Wu, X. *et al.* (2017) *ALTERATIONS OF GUT MICROBIOME IN RHEUMATOID ARTHRITIS*, *Osteoarthritis and Cartilage*. doi: 10.1016/j.joca.2017.02.484.

Xie, Q., Wang, S.-C. and Li, J. (2012) 'Interleukin 22, a potential therapeutic target for rheumatoid arthritis.', *The Journal of rheumatology*. The Journal of Rheumatology, 39(11), pp. 2220; author reply 2221. doi: 10.3899/jrheum.120757.

Yacoub, R. *et al.* (2016) 'Association between probiotic and yogurt consumption and kidney disease: Insights from NHANES', *Nutrition Journal*. doi: 10.1186/s12937-016-0127-3.

Yago, T. *et al.* (2017) 'IL-23 and Th17 Disease in Inflammatory Arthritis.', *Journal of clinical medicine*. Multidisciplinary Digital Publishing Institute (MDPI), 6(9). doi: 10.3390/jcm6090081.

Yanagihara, Y. *et al.* (1999) 'Natural killer (NK) T cells are significantly decreased in the peripheral blood of patients with rheumatoid arthritis (RA)', *Clinical and Experimental Immunology*. doi: 10.1046/j.1365-2249.1999.01018.x.

Yang, L. *et al.* (2016) 'A Possible Role of Intestinal Microbiota in the Pathogenesis of Ankylosing Spondylitis', *International Journal of Molecular Sciences*. MDPI AG, 17(12), p. 2126. doi: 10.3390/ijms17122126.

Yang, Y. J. and Sheu, B. S. (2012) 'Probiotics-Containing Yogurts Suppress Helicobacter pylori Load and Modify Immune Response and Intestinal Microbiota in the Helicobacter pylori-Infected Children', *Helicobacter*. doi: 10.1111/j.1523-5378.2012.00941.x.

Yap, H.-Y. *et al.* (2018) 'Pathogenic Role of Immune Cells in Rheumatoid Arthritis: Implications in Clinical Treatment and Biomarker Development', *Cells*. MDPI AG, 7(10), p. 161. doi: 10.3390/cells7100161.

Yasumoto, S., Imayama, S. and Hori, Y. (1995) 'Increased Serum Level of Interleukin-6 in Patients with Psoriatic Arthritis and Thrombocytosis', *The Journal of Dermatology*. John Wiley & Sons, Ltd (10.1111), 22(10), pp. 718–722. doi: 10.1111/j.1346-8138.1995.tb03908.x.

Yatsunenko, T. *et al.* (2012) 'Human gut microbiome viewed across age and geography', *Nature*, pp. 222–227. doi: 10.1038/nature11053.

Yeoh, N. *et al.* (2013) 'The Role of the Microbiome in Rheumatic Diseases', *Current Rheumatology Reports*. Current Science Inc., 15(3), p. 314. doi: 10.1007/s11926-012-0314-y.

Yilmaz, M., Arslan, F. and Mert, A. (2014) 'Community Acquired Chronic Arthritis due to Pseudomonas aeruginosa in a Previously Healthy Pregnant Woman.', *Case reports in infectious diseases*. Hindawi, 2014, p. 272306. doi: 10.1155/2014/272306.

Yinshi Yue, Y. Y. (2013) 'Microbial Infection and Rheumatoid Arthritis', *Journal of Clinical & Cellular Immunology*. OMICS Publishing Group, 04(06). doi: 10.4172/2155-9899.1000174.

Yoshida, A. *et al.* (2001) 'Immunodominant Region of Actinobacillus actinomycetemcomitans 40-kilodalton Heat Shock Protein in Patients with Rheumatoid Arthritis', *Journal of Dental Research*. SAGE PublicationsSage CA: Los Angeles, CA, 80(1), pp. 346–350. doi: 10.1177/00220345010800010901.

Yoshida, S. *et al.* (1999) 'Involvement of thioredoxin in rheumatoid arthritis: Its costimulatory roles in the TNF-α-induced production of IL-6 and IL-8 from cultured synovial fibroblasts', *Journal of Immunology*.

Yoshida, Y. and Tanaka, T. (2014a) 'Interleukin 6 and rheumatoid arthritis.', *BioMed research international*. Hindawi, 2014, p. 698313. doi: 10.1155/2014/698313.

Yoshida, Y. and Tanaka, T. (2014b) 'Interleukin 6 and rheumatoid arthritis.', *BioMed research international*. Hindawi Limited, 2014, p. 698313. doi: 10.1155/2014/698313.

Yoshino, S. and Ohsawa, M. (2000) 'The role of lipopolysaccharide injected systemically in the reactivation of collagen-induced arthritis in mice', *British Journal of Pharmacology*. John Wiley and Sons Inc., 129(7), pp. 1309–1314. doi: 10.1038/sj.bjp.0703166.

Yousfi, K. *et al.* (2017) 'Current trends of human infections and antibiotic resistance of the genus Shewanella', *European Journal of Clinical Microbiology & Infectious Diseases*, 36(8), pp. 1353–1362. doi: 10.1007/s10096-017-2962-3.

Yurkovetskiy, L. *et al.* (2013) 'Gender bias in autoimmunity is influenced by microbiota', *Immunity*. doi: 10.1016/j.immuni.2013.08.013.

Zabotti, A. *et al.* (2016) 'Differentiation between early rheumatoid and early psoriatic arthritis by the ultrasonographic study of the synovio-entheseal complex of the small joints of the hands', *Clinical and Experimental Rheumatology*.

Zambon, J. J. *et al.* (1996) 'Cigarette Smoking Increases the Risk for Subgingival Infection With Periodontal Pathogens', *Journal of Periodontology*. doi: 10.1902/jop.1996.67.10s.1050.

Zhang, X. *et al.* (2015) 'The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment', *Nature Medicine*, 21(8), pp. 895–905. doi: 10.1038/nm.3914.

Zhang, Y. *et al.* (2018) 'Crosstalk between gut microbiota and Sirtuin-3 in colonic inflammation and tumorigenesis', *Experimental and Molecular Medicine*. doi:

10.1038/s12276-017-0002-0.

Zhang, Zhengxiao *et al.* (2019) 'Impact of fecal microbiota transplantation on obesity and metabolic syndrome- a systematic review', *Nutrients*. doi: 10.3390/nu11102291.

Zhang, Zhihui *et al.* (2019) 'Shexiang-Wulong Pills Attenuate Rheumatoid Arthritis by Alleviating Inflammation in a Mouse Model of Collagen-Induced Arthritis', *Evidence-Based Complementary and Alternative Medicine*. Hindawi, 2019, pp. 1–9. doi: 10.1155/2019/5308405.

Zhao, Q. and Elson, C. O. (2018) 'Adaptive immune education by gut microbiota antigens', *Immunology*, 154(1), pp. 28–37. doi: 10.1111/imm.12896.

Zhao, Y. *et al.* (2018a) 'Detection and characterization of bacterial nucleic acids in culture-negative synovial tissue and fluid samples from rheumatoid arthritis or osteoarthritis patients.', *Scientific reports*. Nature Publishing Group, 8(1), p. 14305. doi: 10.1038/s41598-018-32675-w.

Zhao, Y. *et al.* (2018b) 'Detection and characterization of bacterial nucleic acids in culture-negative synovial tissue and fluid samples from rheumatoid arthritis or osteoarthritis patients', *Scientific Reports*. Nature Publishing Group, 8(1). doi: 10.1038/s41598-018-32675-w.

Zheng, S. *et al.* (2017) 'Clinical Parameters and Gut Microbiome Changes Before and After Surgery in Thoracic Aortic Dissection in Patients with Gastrointestinal Complications', *Scientific Reports*. Nature Publishing Group, 7(1), p. 15228. doi: 10.1038/s41598-017-15079-0.

Zhernakova, A. *et al.* (2016) 'Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity', *Science*. doi: 10.1126/science.aad3369.

Zhou, C. *et al.* (2020) 'Metagenomic profiling of the pro-inflammatory gut microbiota in ankylosing spondylitis', *Journal of Autoimmunity*. Academic Press, 107, p. 102360. doi: 10.1016/j.jaut.2019.102360.

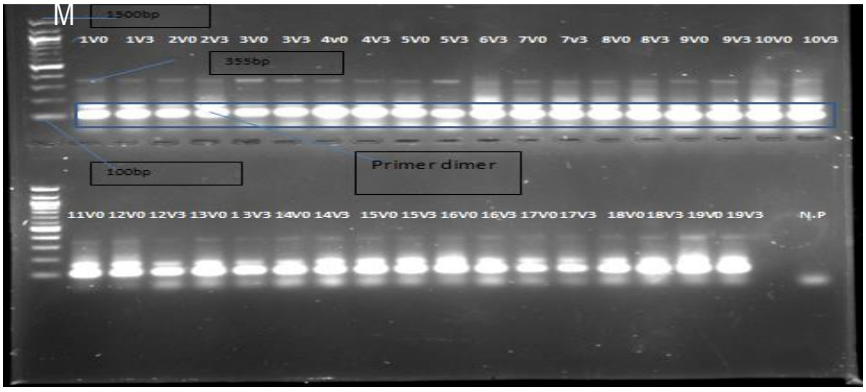
Ziesemer, K. A. *et al.* (2015) 'Intrinsic challenges in ancient microbiome reconstruction using 16S rRNA gene amplification', *Scientific Reports*. Nature Publishing Group, 5(1), p. 16498. doi: 10.1038/srep16498.

Zimmermann, M. *et al.* (2019) 'Mapping human microbiome drug metabolism by gut bacteria and their genes', *Nature*. Nature Publishing Group, 570(7762), pp. 462–467. doi: 10.1038/s41586-019-1291-3.

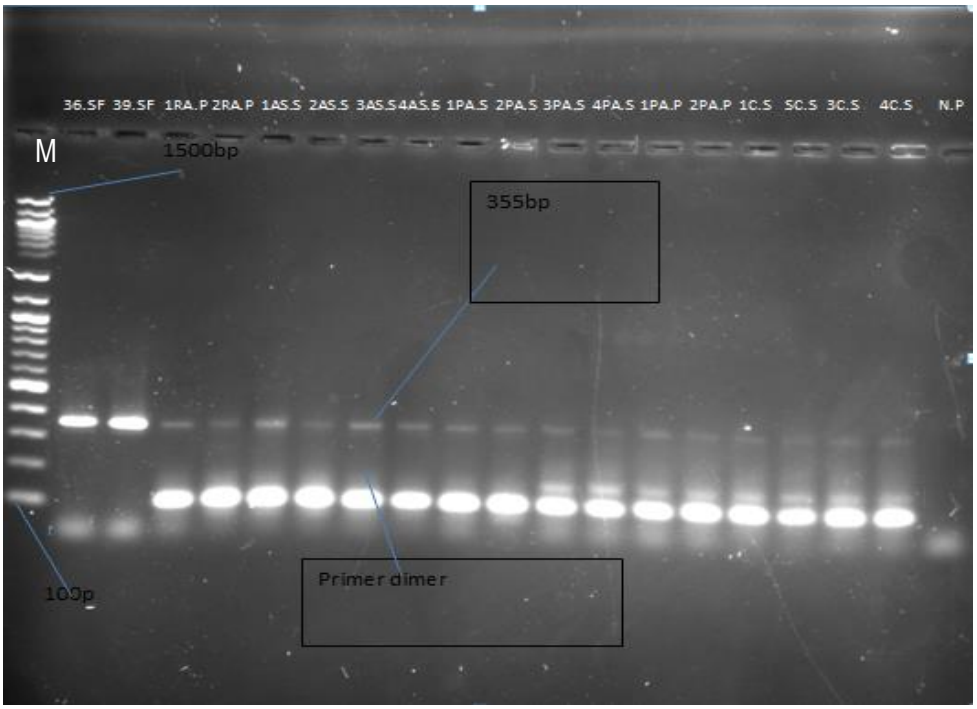
Ziolkowska, M. *et al.* (2000) 'High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism.', *Journal of immunology (Baltimore, Md. : 1950)*.

8 Appendices

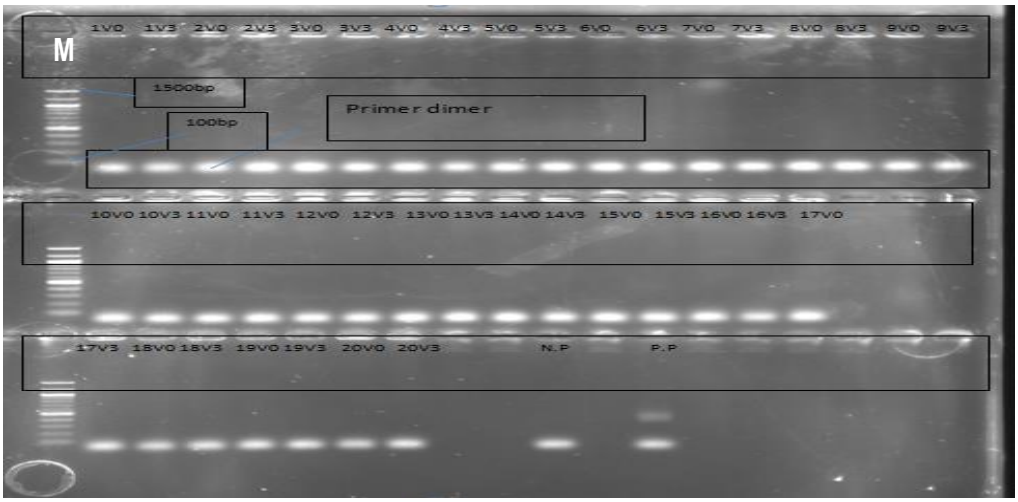
A- **Detection 16S rRNA by 16s rRNA v4 with XT tag Illumina primers in RA serum samples obtained from Heywood hospital cohort.**



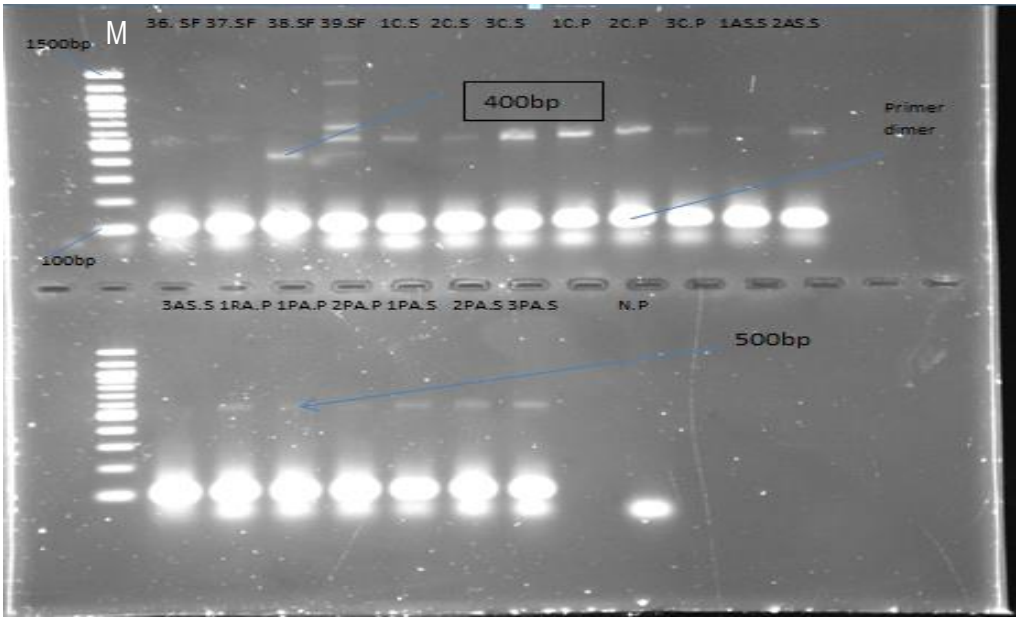
B- **Detection 16SrRNA by 16s rRNA v4 with XT tag Illumina primers in synovial fluid and plasma from RA, from serum samples from ankylosing spondylitis (AS) patients, serum and plasma samples from psoriatic arthritis (PA) patients, and serum from healthy control subjects obtained from sera lab cohort.**



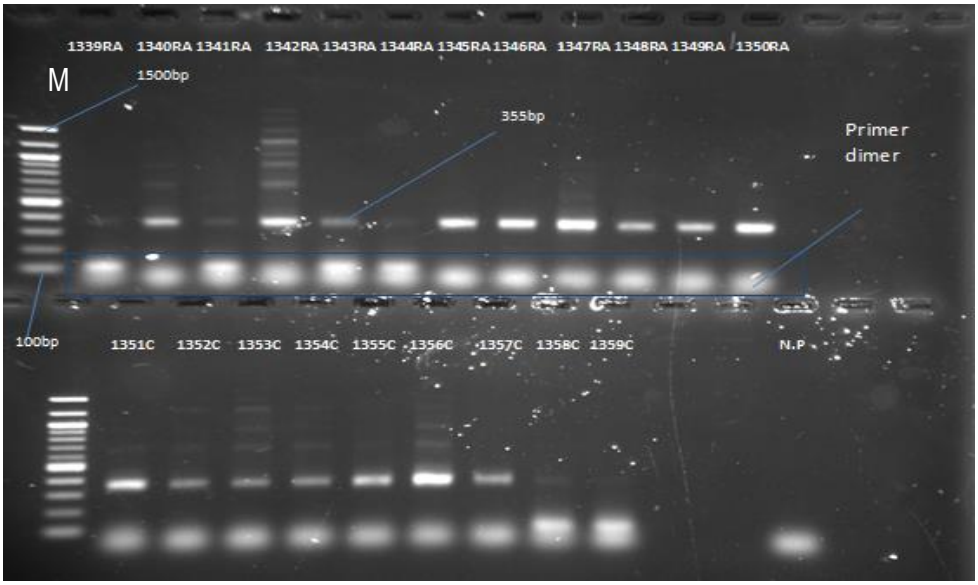
C- ITS2 primers did not detect fungal DNA in RA serum samples



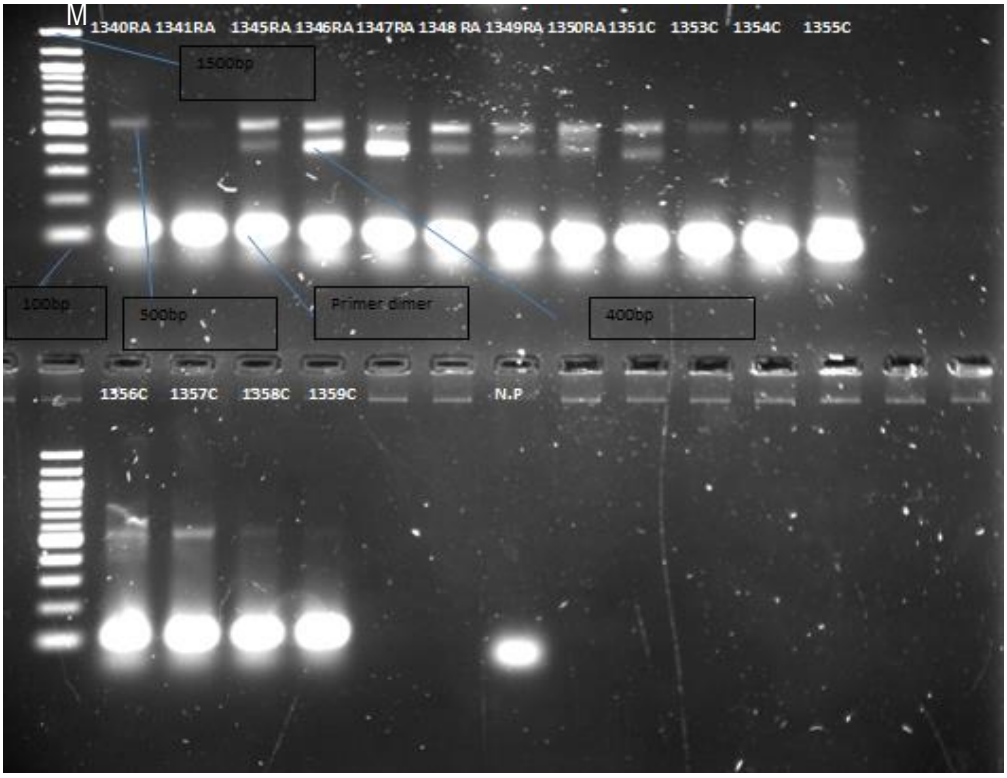
D- Detection ITS2 by ITS2 with XT tag Illumina primers in the samples obtained sera lab cohort (synovial fluid, plasma, and serum) which taken from diseased and healthy control subjects



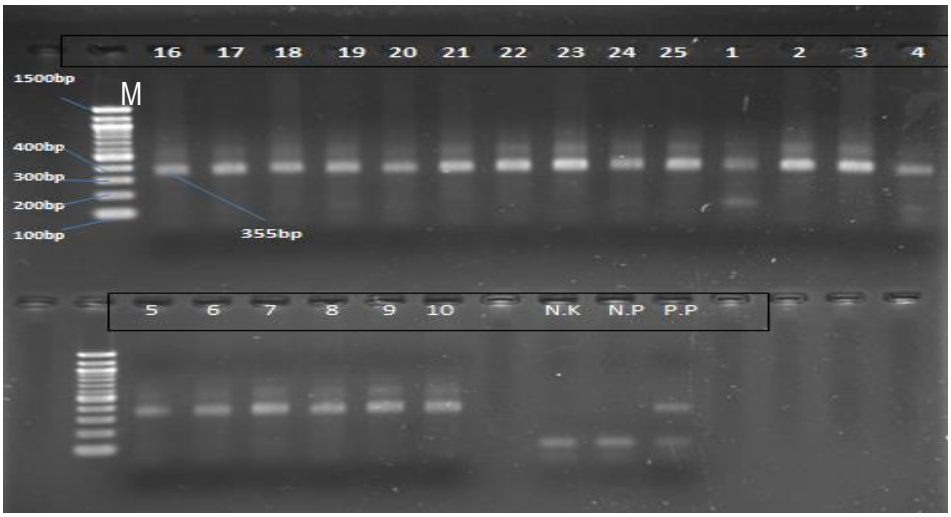
E- Detection 16SrRNA by 16s rRNA v4 with XT tag Illumina primers in the synovial fluid of RA patients and healthy controls samples obtained from Sera lab cohort



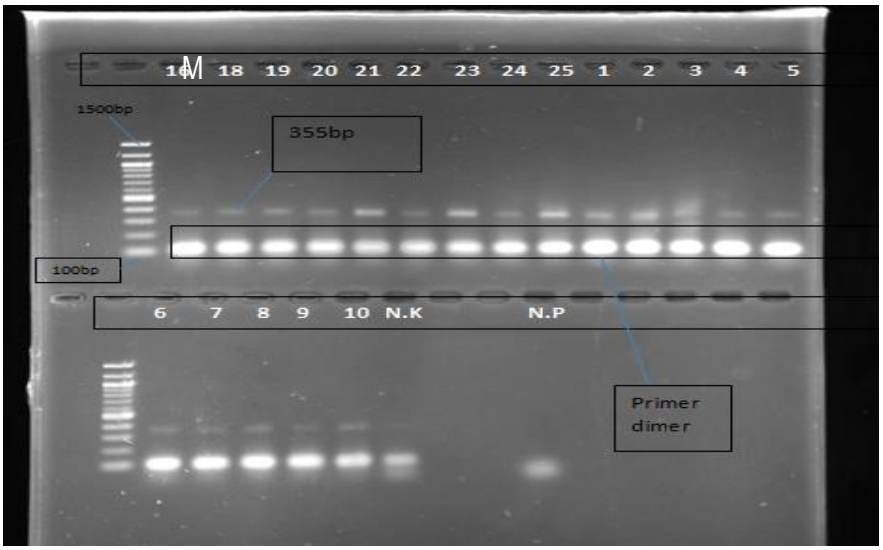
F- Detection ITS2 by ITS2 with XT tag Illumina primers in the synovial fluid of RA patients and healthy control samples obtained from Sera lab cohort



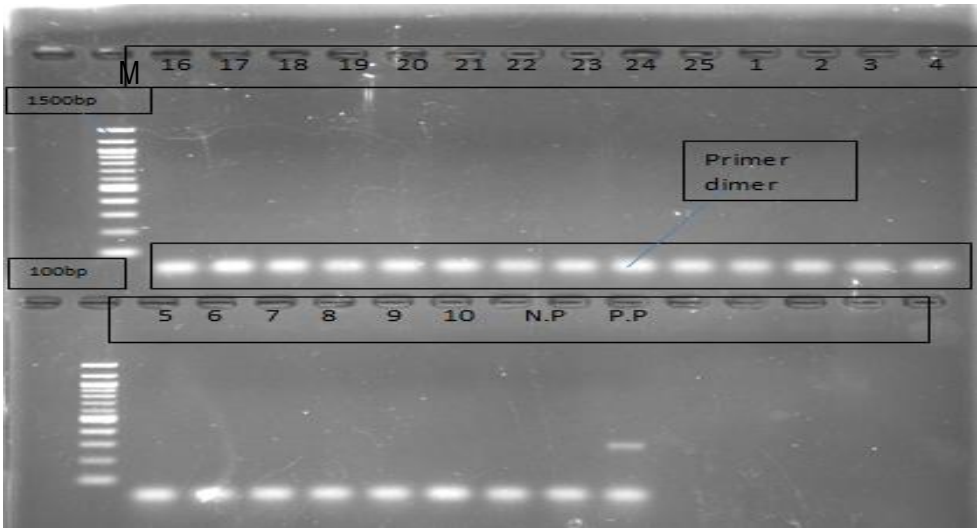
G- Detection 16S rRNA by 16s rRNA v4 with XT tag Illumina primers in the stool of CIA and healthy controls samples obtained from Axis Bioservices



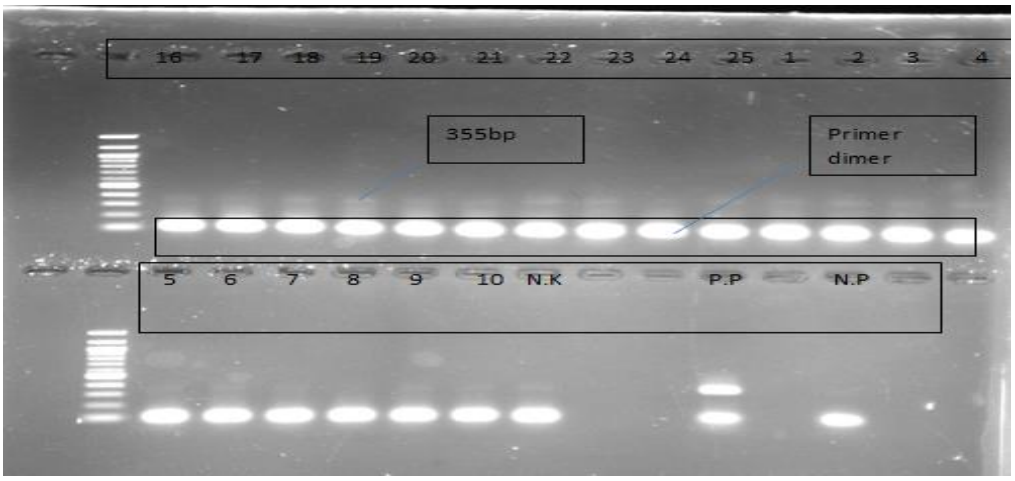
H- Detection 16S rRNA by 16s rRNA v4 with XT tag Illumina primers in the urine of CIA, healthy controls, samples obtained from Axis Bioservices.



I- 16s rRNA v4 primers did not see bacterial DNA in the synovial fluid of CIA and healthy controls subject



J- Detection 16S rRNA by 16s rRNA v4 with XT tag Illumina primers in the blood of CIA, healthy controls, samples obtained from Axis Bioservices



κ- Detection 16S rRNA by 16s rRNA v4 with XT tag Illumina primers in the serum of CIA, and healthy controls samples obtained from Axis Bioservices

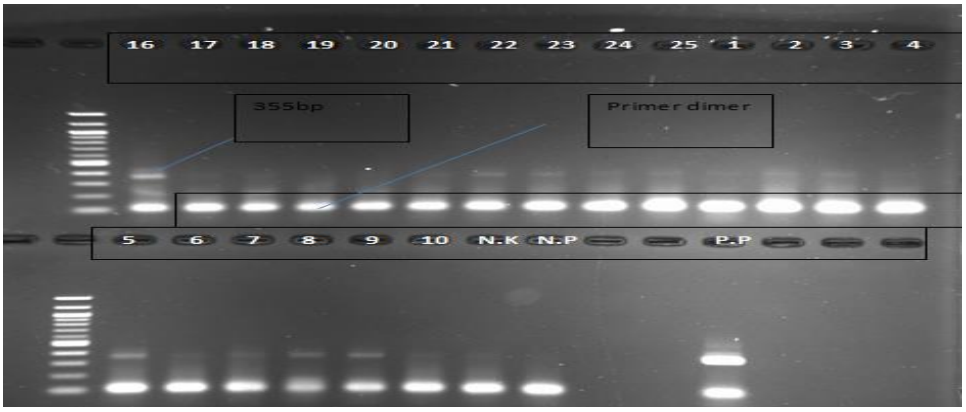


Figure 8.1 High throughput amplicon (16S and ITS) sequencing was used to identify the bacterial and fungal DNA in the biological samples of human and mouse subjects, Lanes M, represents a molecular weight marker (100bp). A, B Bacterial DNA was detected by PCR amplification in serum samples from R.A., ankylosing spondylitis, R.A. synovial fluid, and serum and plasma from psoriatic arthritis patients, and serum from healthy controls, for **A,** lane 1V0- 19V3 represent R.A. serum, lane N.P represents negative PCR reaction, for B lane 36 S.F and 39 S.F. represent R.A. synovial fluid, lane 1RA.P, and 2RA.P represent R.A. plasma, the lane from 1AS.S- 4AS.S represent serum samples from ankylosing spondylitis, the lane from 1PA.S to 2PA.S represent serum and plasma samples from psoriatic arthritis patients, lane 1C.S - 4C.S represent serum samples from healthy controls, lane N.P represent negative PCR reaction. The results showed a successful bacterial 16S amplification in these samples, with expected bands =355bp. **C**, Lane from 1V0-20v3 represent the serum samples from R.A., lane N.P. and P.P. represent negative and positive PCR reaction, respectively. Our finding showed that ITS2 fungal `was not seen in the R.A. serum samples. **D,** lane 36 S.F to 39 S.F. represent R.A. synovial fluid, lane 1C.S to 3C. P represents serum and plasma from healthy controls, the lane from 1AS.S- 3AS.S represent serum samples from ankylosing spondylitis, lane 1RA.P represent R.A. plasma, the lane from 1PA.P to 3PA.S represent serum and plasma samples from psoriatic arthritis patients, lane N.P represents negative PCR reaction. The results identified successful fungalITS2 amplification in these samples; with expected bands from 400bp to 500bp. **E,** the lane from 1339RA to 1350RA represent the synovial fluid from R.A. patients, lane 1351C to 1359C represent the synovial fluid from a healthy subject, lane N.P represents negative PCR reaction. The results identified successful 16S amplification in these samples; with expected band 355bp. **F,** the lane from 1340RA to 1350RA represent the synovial fluid from R.A. patients, lane 1351C to 1359C represent the synovial fluid from a healthy subject, lane N.P represents negative PCR reaction. The results identified successful ITS2 amplification in these samples; with expected band 400bp to 500bp. From **G-K**, the detection of 16S rRNA in the stool, urine, synovial fluid, blood and serum of CIA and controls, lanes from 16 to 25 represent CIA samples, lane 1 to 10 represent controls samples. From **G** (stool samples) the results showed the successful 16S amplification in these samples with no amplification in N.K. represent negative kit control. For **H, J and K** (urine, blood, and serum), the results found the successful 16S amplification in these samples with amplification in N.K. represent negative kit control. **I**, 16S was not detected in the synovial fluid of CIA and controls subjects.