The clinical and biological effects of the use of probiotic VSL#3 in patients with oral lichen planus

By

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I, Erni Marlina confirm that the work presented in this thesis is my own. Where information has been derived from other source, I confirm that this has been indicated in the thesis.
ALHAMDULILLAH
Abstract

Oral lichen planus (OLP) is an inflammatory - autoimmune disease of the oral cavity with unknown aetiology. This disease accounts for almost 40% of the visits yearly to the Eastman Dental Hospital. Currently, there is no curative therapy and the management is aimed at reducing painful symptoms, which is typically achieved through healing of mucosal erosion and ulceration. A novel therapeutic approach could be the poly-biotic VSL#3 that is known to have anti-inflammatory and wound healing properties in diseases of the gastrointestinal tract. In this thesis, we hypothesise that the poly-biotic VSL#3 might have a potential therapeutic effect on oral lichen planus (OLP) lesion.

In vitro experiments were focused on isolating, identifying, and characterizing the single species contained in VSL#3 and comparing their immunomodulatory properties to the whole mixture. I used both the human monocytic cell line THP-1 and the human oral epithelial line MOE1a to gain an understanding of how probiotics influence the immune response to pro-inflammatory stimuli such as E. coli and interferon-γ. I developed an oral epithelial wound healing assay to determine the effects of VSL#3 on the speed of healing and cell morphology. In vivo studies were performed to A. Profile the cytokine levels and neutrophil reactive oxygen species (ROS) generation in patients with OLP and compare between different disease phenotypes (n=80) and healthy control (HC) subjects (n=44). B. Setup and ran the first ever double-blind, randomized, placebo-controlled, feasibility two arm study on the treatment of OLP patients (n=30) with VSL#3. In the clinical trial I quantified a range of clinical and mechanistic parameters such as pain score, oral disease severity score, quality of life, serum and saliva cytokines level, metagenomics changes, and most importantly the safety, tolerability, and acceptance of the participants toward the highly concentrated poly-biotic VSL#3.

The initial in vitro results provide assurance that at a cellular level VSL#3 was non-toxic and unable to elicit a strong immunological response in THP-1 or MOE-1a cells. In fact, VSL#3 and the isolated single species were all capable of reducing the immune response of both THP-1 and MOE-1a cells upon E. coli and IFN-γ stimulation. A beneficial effect of VSL#3 on wound healing was also observed and provided encouragement for the therapeutic potential of this probiotic in OLP. Cytokine profiling of OLP patients revealed an elevation in pro-inflammatory cytokines, which differed depending on disease phenotype. The release of CXCL10 and IFN-γ was higher in erosive OLP, which is the most aggressive form of the disease. The clinical trial was successfully completed and analysed within the study period. The pilot data demonstrated the safety and acceptability of the VSL#3 in the OLP patients (compliance 100%). The overall trend was highly suggestive of a beneficial effect of VSL#3 on OLP with a trend for a reduction in mean site score, CXCL10/IFN-γ levels in saliva and an improvement in quality-of-life score. Taken together, this study supports the use of VSL#3 and may be probiotics in general as an adjunct therapy for OLP.
Impact Statement

Probiotics have long been known to have a beneficial effect on human health. They have been shown to possess anti-inflammatory properties, aid in the digestion of food and metabolise essential amino acids and vitamins. The World Health Organisation have defined a probiotic as a living microorganism that when administered in adequate amounts will confer a health benefit to the host. A literature search revealed that Pubmed has indexed more than 24,000 research articles which contain the term probiotics in the title or abstract. Those publications report a huge range of benefits attributed to probiotics in population with a different underlying systemic background.

Specific strains of probiotics have been attributed different beneficial effects and this has resulted in the development of products that contain a cocktail of different bacteria, which is believed to improve effectiveness over a single strain. One of these mixtures is the poly biotic VSL#3, which contains 8 different species. The poly biotic VSL#3 has been investigated intensively for a wide range clinical situations, but is most commonly used in inflammatory bowel diseases, ileoanal pouchitis and ulcerative colitis. The exact mechanism of action is still unclear, but some articles have reported a potential role in modulating gut barrier functions, inhibiting pro-inflammatory cytokines and reducing associated-severity of disease leading to an improvement in quality of life. The work I present in this thesis investigates the potential benefit of VSL#3 and probiotics in oral inflammation and assessed the feasibility of using this poly-biotic in the treatment of oral lichen planus. Oral lichen planus is a chronic inflammatory disease of the oral mucosa with an unknown aetiology. This global disease affects between 0.2 to 4% of the population and locally contributes to approximately 40 percent of all appointments at the Eastman Dental Hospital Oral Medicine Unit (~1,000 clinical appointments per year). The disease is associated with pain, long standing ulceration, frequent flare up, and increased risk of oral cancer, which currently is incurable. Corticosteroids along with other immunosuppressant such as tacrolimus and pimecrolimus are the standard treatment which is beneficial but not curative and can have a number of adverse side effects. A safer alternative has long been sought by physicians and is desperately needed by the patients who suffer from frequent flare ups, which tends to lead to a significant loss in quality of life.
My findings provide a potential novel alternative adjunct therapy for this disease. My double-blind randomized placebo-controlled trial generated basic pilot feasibility data which will be used to develop a larger definitive trial on the use of probiotics in oral lichen planus. Additionally, I have data which demonstrates that probiotics can directly influence the oral epithelial cells and reduce inflammation and speed up wound healing, which could be relevant to a number of different diseases. The assays I have developed during my PhD will be used to screen alternative probiotics and pharmaceutical drugs in future studies. In relation to oral lichen planus, I demonstrated that CXCL10 and IFN-γ may have an important role in the disease pathogenesis and could be a potential target for other alternative treatment or act as a biomarker for disease activity. These findings will be of considerable interest to pharmaceutical companies that develop treatments for inflammatory diseases and diagnostic tests.

In general, the work will be of interest to other investigators in the field of oral medicine and mucosal immunology. It is possible that VSL#3 or similar products may be of benefit to other inflammatory diseases in oral environment. Finally, and most importantly, the work may provide patients with a safe alternative maintenance therapy for their chronic inflammatory disease.
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### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCS</td>
<td>Advisory committee on borderline substance</td>
</tr>
<tr>
<td>Ag</td>
<td>Silver</td>
</tr>
<tr>
<td>AJs</td>
<td>Adherent junctions</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BNF</td>
<td>British national formulary</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine pituitary extract</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSM</td>
<td>Bifido selective medium</td>
</tr>
<tr>
<td>CABRIO</td>
<td>Clinical and Biological</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDR</td>
<td>Clinical data repository</td>
</tr>
<tr>
<td>COMDQ</td>
<td>Chronic oral mucosal disease questioners</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRF</td>
<td>Case report form</td>
</tr>
<tr>
<td>DAMPS</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DK-SFM</td>
<td>Defined keratinocytes-Serum free medium</td>
</tr>
<tr>
<td>DM2</td>
<td>Diabetes mellitus type 2</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemiluminescent</td>
</tr>
<tr>
<td>EDH</td>
<td>Eastman Dental Hospital</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune assay</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ESS</td>
<td>Escudier’s scoring system</td>
</tr>
<tr>
<td>EVOM2</td>
<td>Epithelial volt/ohm meter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal buffer serum</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Foxhead box protein 3</td>
</tr>
<tr>
<td>GM</td>
<td>Geometric mean</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HClO</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HE</td>
<td>Hepatoencephalopathy</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HkEc</td>
<td>Heat killed- <em>E. coli</em></td>
</tr>
<tr>
<td>HO</td>
<td>Hydroxyl radicals</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish-peroxidase streptavidin</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>H₂O</td>
<td>Hydrogen oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel diseases</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-..</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin receptor antagonist</td>
</tr>
<tr>
<td>IRAS</td>
<td>Integrated research approval system</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon response factor-3</td>
</tr>
<tr>
<td>ISAPP</td>
<td>International scientific association for probiotic and prebiotic</td>
</tr>
<tr>
<td>ITS 1&amp;2</td>
<td>Ribosomal-internal transcribed spacer region</td>
</tr>
<tr>
<td>ITT</td>
<td>Intention to treat</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinases</td>
</tr>
<tr>
<td>JAM-A</td>
<td>Junction adhesion molecule A</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen 1</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal association of lymphoid tissue</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complexes</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Moloney murine leukemia virus reverse transcriptase</td>
</tr>
<tr>
<td>MRS</td>
<td>De Man-Rogosa Sharpe</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso scale discovery</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NHS</td>
<td>National health system</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleat acid</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso scale discovery</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>pNRS</td>
<td>Pain numeric rating scale</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleat acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>ODSS</td>
<td>Oral disease severity score</td>
</tr>
<tr>
<td>OHIP</td>
<td>Oral health impact</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>OLP</td>
<td>Oral lichen planus</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase</td>
</tr>
<tr>
<td>PPRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>RAS</td>
<td>Recurrent aphthous stomatitis</td>
</tr>
<tr>
<td>REC</td>
<td>Research ethic committee</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROO</td>
<td>Peroxyl radical</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Riboxy nucleat acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAP</td>
<td>Statistical analysis plan</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokines signalling 1</td>
</tr>
<tr>
<td>STATs</td>
<td>Signal transducer and activator of transcriptions proteins</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll/interleukin domain-containing adapter</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UCLH</td>
<td>University college london hospital</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular adhesion molecule-1</td>
</tr>
<tr>
<td>VSL#3</td>
<td>Verseley (name's of patented company of multi-probiotic)</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
</tr>
<tr>
<td>ZOT</td>
<td>Zonula occluding toxin</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Oral lichen planus

1.1.1 Definition

Lichen Planus (LP) is a T-cell mediated chronic inflammatory mucocutaneous disease causing long-standing cutaneous and mucosal manifestation.\(^{(1-7)}\) LP can affect all areas of stratified epithelial, mainly the skin and oral mucosa, but can involve other areas such as vulvar and vaginal mucosa, glans penis, hair follicles (lichen planopilaris, resulting in scarring alopecia alopecia), nails and rarely the eyes, urinary tract, nasal mucosa and larynx.\(^{(6, 9)}\) LP mostly affects the oral mucosa in absence of skin lesions, and about 60% of affected cutaneous patients also having oral lesions called oral lichen planus (OLP).\(^{(10)}\) While cutaneous lesions of LP can be self-limiting and pruritic, oral lesions are commonly chronic, non-remissive and can be a source of morbidity.\(^{(9)}\) OLP is more prevalent in middle-aged females. The typical age of presentation is 30 to 60 years of age, although younger adults and children may be affected, with female to male ratio approximately 1.4:1.\(^{(11, 12)}\)

Long standing use of medications, lesion recurrence and adverse side effects of therapy in OLP patients eventually leads to a significant reduction in a patient quality of life.\(^{(20, 21)}\) Based on the Dermatology Life Quality Index (DLQI), OLP patients showed significant DLQI score when compared with cutaneous LP patients,\(^{(22)}\) while the Oral Health impact (OHIP) showed the significant psychological discomfort and social disability in OLP patients. Increased visual analogue scale score (VAS) was associated with poor oral health that also related to quality of life (QoL) of patients with OLP.\(^{(23)}\) There is also an increased risk of
oral squamous cell carcinoma development in OLP and has been classified by WHO as a potential malignant disorder.\(^2\) There is currently no cure for OLP and treatment is based on disease management and maintenance of remission typically through the use of corticosteroids.

1.1.2 Aetiopathogenesis

The aetiology of OLP is unknown, however some authors have suggested potential triggers and contributing factors in OLP, including:

1) Local and systemic inducers of cell-mediated hypersensitivity
2) Stress
3) Autoimmune response to epithelial antigens
4) Microorganisms\(^{10}\)

Other pathology condition resembles oral lichen planus both clinically and histologically termed as lichenoid lesions.\(^8\) The disease presented in several clinical settings. Hypersensitivity responses generally align with lichenoid mucositis, such as those seen with local reactions to dental restorative materials or flavouring agents, or adverse responses to systemic medications.\(^8\) It was reported that approximately 3-5% incidence of oral lichenoid lesions-type due to dental amalgam, and 37-78% of those have also hypersensitivity to mercury and/or silver.\(^{13}\)

Oral lichen planus diagnosis requires clinicopathology of presence more or less symmetrical lesion in conjunction with a well-defined, band-like lymphocytic infiltrate, and signs of liquefactive degeneration of the basal epithelial cells.\(^{14}\) The psychological stress been reported as a contributing factor to OLP. Preda et al, described oral mucosal area as a primary erogenic zone which is massively complex and vulnerable and very reactive to certain psychological influences.\(^{15-17}\)
The psychological effect termed as psychosomatic diseases. The suggestion agreed with the Cornell Medical Index (CMI) test which demonstrated significant differences between the psychological constitutions of OLP patients compared to a control group.\textsuperscript{(15, 18)}

Furthermore, Chiappelli \textit{et al} reported differences in psychoimmune interactions between patients afflicted with non-erosive OLP lesion and those with erosive OLP lesions.\textsuperscript{(18, 19)} Cortisol is the key hormone in the stress response and is related to the development of depression. Significantly higher levels of cortisol was identified in OLP patients leading to conclusion of relation to stress condition.\textsuperscript{(20, 21)} Some authors even suggest psychotherapy in OLP management may be of some benefit.\textsuperscript{(15)} However, contrary with previous studies, two others authors demonstrated that no relevant connection between stress and OLP could be identified.\textsuperscript{(15, 22)}

Definitive evidence for autoimmunity in OLP has not been demonstrated, although a number of studies point to dysregulated immune responses, which allow for the possibility of autoimmunity.\textsuperscript{(23)} It is stated that there is a consensus regarding chronic, cell mediated, immune damage to basal keratinocyte that occurs in OLP.\textsuperscript{(24, 25)} Further investigations revealed that the most abundant inflammatory cells within the legion belonged to the T lymphocyte (T cells) lineage, with a predominant cytotoxic T cells component in close proximity to the epithelial basement membrane.\textsuperscript{(26)} Immune cells, including the cytotoxic lymphocytes generating communication by the cell to cell contacts known as immune synapses. In addition to this internal communication, the immune system makes use of the synapse process for direct attack on infected and cancerous cells. This formation allows killer cells to overcome the challenge of specifically eliminating ‘dangerous’
cells whilst leaving healthy cells unaffected. It is only after the establishment of the
focused synapse interface that cytotoxic T lymphocytes (CTLs) and natural killer
cells deliver a cocktail of cytotoxic substance from specialised secretory lysosomes
(cytolytic granules) to destroy the target.\(^{(27, 28)}\) in OLP the effector cells causing
generation or destruction of keratinocytes are the CTLs.\(^{(25, 29)}\)

Several microbial agents have been investigated for their possible role in
OLP,\(^{(30-32)}\) of which hepatitis C virus has thus far emerged as the only
microorganism with a convincing association, and that only in some geographic
regions. Important studies of other mucosal factors, such as the oral microflora
and receptors that control antimicrobial responses, are beginning to emerge.\(^{(33-38)}\)
whether the destruction of keratinocytes in OLP occurs due to autoimmune
reactivity to an endogenous peptide or due to a dysregulated response to an
exogenous antigen has so far proved enigmatic.

**1.1.3 Clinical presentation and Diagnosis**

The clinical presentation of OLP is most commonly characterised by the
appearance of white or red lesions, multifocal, of symmetric distribution, being
classified as reticular/papular, atrophic (erythematous), erosive (ulcerative),
plaque-like and bullous  (Figure 1.1).\(^{(39)}\) General definitions of the phenotype
covered in this thesis refer to the Eisen criteria\(^{(40)}:\)

1. A Wickham’s striae phenotype with interlacing of keratotic, pinhead sized,
   white, slightly elevated papules or discrete plaque-like area of arranged in
   reticular web-like feature.
2. Erythematous phenotype refers to superficial reddening of the oral mucosal
   without any loss of mucosal layer. It could be surrounded by fine white
   striae.
3. Erosive phenotype presents as either atrophic red areas within the white lesions or well-defined ulceration. Desquamative gingivitis and bullous feature are also included in this phenotyping, due to the clinical characteristic of ulceration/erosive features whenever the bullae is broken.

![Figure 1.1](image)

Figure 1.1 Oral lichen planus phenotype. A, B, and D. White homogenous reticular lesions. A. displayed classical web-like appearance (Wichkam's striae) while B. is more papular dense, and C displayed plaque-like lesion (Courtesy of Greenberg, 2008). D, E, and F are the erosive/ulcerative lesions with heterogenous white plaque or reticular area (D and E are courtesy of Sugerman, 2002 and E. Courtesy of Sara-Lia, 2019). G. Typical reticular striae with secondary melanosis. H. Interlacing striae with annular keratotic striae (reticular one). I. Generalise desquamasi with some fragility. (G, H and I are courtesy of Sugerman, 2002).

OLP mostly presents on the buccal mucosa, gingiva, and tongue and manifests in the form of bilateral and symmetric lesions. Subjectively, OLP lesions usually persist for many year, fluctuating between exacerbation and quiescence. Increased pain and sensitivity are most reported during exacerbation periods, which clinically would be usually associated with ulcerated or erosive or
more erythema lesions. Whenever the extent of erythema or ulceration decreases the pain and discomfort would also reduce.\textsuperscript{[4, 42]}

Diagnosis is usually based on clinical examination and confirmed with histopathology. Histopathological diagnostic criteria have been defined by WHO and include hyperkeratosis, acanthosis, basal layer degeneration/vacuolization, stromal apoptotic keratinocytes, and band-like sub-epithelial lymphocytic infiltrate (Figure 1.2). The presence of fibrinogen and complement at the basement membrane zone can be demonstrated through direct immunofluorescence.\textsuperscript{[43]}

The WHO criteria (Table 1.1.) guide the clinician to the challenges in making the diagnosis of OLP, mainly in differentiated OLP with other disorders that clinically and/or histopathologically resemble OLP such as mucous membrane pemphigoid, lichen planus pemphigoid, chronic graft-versus-host disease, chronic ulcerative stomatitis, oral lichenoid drug reactions, oral lichenoid contact hypersensitivity reactions, lupus erythematosus, and proliferative verrucous leucoplakia.\textsuperscript{[10]} The final diagnosis of OLP should feature classical histopathological characteristics such as the presence of an infiltrate predominantly consisting of lymphocytes distributed as a band in the subepithelial region, lymphocyte exocytosis, basal keratinocyte liquefaction and should be associated with the clinical characteristics\textsuperscript{[39]}. 
**Figure 1.2.** Clinical and Histology features of the oral mucosa. Normal oral mucosa (I) IA. Stratified squamous epithelium overlies the papillary (p) and reticular (R) lamina propria and submucosa (sm), with adipose tissue and lobules of minor salivary glands (msg). IB. Downward, undulating epithelial rete pegs (arrows) interdigitate with the apical uppermost (*) aspect of the papillary lamina propria. IC. Note the regimented-appearing epithelial basal cells. Stem cells (*). Small blood vessels (cp) and delicate collagen fibers are present in the papillary lamina propria, as contrasted with the ticker collagen fibers of the reticular lamina propria (R with arrows) that run parallel to the epithelial surface. Oral lichen planus (II) IIA. Oral mucosal stratified squamous epithelium exhibits a thickened surface layer of parakeratin, saw-tooth rete ridge morphology a thin eosinophilic band adjacent to the basal cell layer, and a dense band-like chronic inflammatory cell infiltrate in the superficial lamina propria. IIB. A dense predominantly lymphocytic infiltrate is situated in the lamina propria abutting oral mucosal stratified squamous epithelium. Hydrophobic degeneration in basal cell is apparent. Dissolution of the basement membrane is also seen. IIC. Lymphocyte-mediated injury of oral mucosal stratified squamous epithelium, with keratinocyte apoptosis represented as a colloid (Civatte) body (arrow). (Normal oral mucosa histology adapted from Eisenberg E. et al. 2020, OLP histology adapted from Cheng Y.S et al, 2016).
Table 1.1 OLP diagnosis based on WHO 1978 criteria and modification criteria 2003(10)

<table>
<thead>
<tr>
<th>WHO Criteria</th>
<th>Modified WHO criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical criteria</td>
<td>Bilateral, more or less symmetric lesions</td>
</tr>
<tr>
<td>- Usually multiple, and often symmetric in distribution</td>
<td>- Erosive, atrophic, bullous, and plaque-type lesions are only accepted as a subtype in the presence of reticular lesions elsewhere in the oral mucosa</td>
</tr>
<tr>
<td>- White papular, reticular (lace-like network of slightly raised graywhite lines), annular, or plaquetype lesions</td>
<td>- Lace-like network of slightly raised gray-white lines (reticular pattern)</td>
</tr>
<tr>
<td>- White lines radiating from the papules</td>
<td></td>
</tr>
<tr>
<td>- Atrophic lesions with or without erosion</td>
<td></td>
</tr>
<tr>
<td>- Bullae are rare</td>
<td></td>
</tr>
<tr>
<td>Histopathologic criteria</td>
<td>Well-defined, band-like zone of cellular infiltration consisting mainly of lymphocytes and confined to the superficial lamina propria</td>
</tr>
<tr>
<td>- Orthokeratosis or parakeratosis</td>
<td>Liquefaction degeneration in the basal cell layer</td>
</tr>
<tr>
<td>- Epithelial thickness varies, saw-tooth rete ridges sometimes seen</td>
<td>Absence of epithelial dysplasia</td>
</tr>
<tr>
<td>- Civatte bodies in the basal layer of the epithelium or superficial lamina propria</td>
<td></td>
</tr>
<tr>
<td>- A narrow band of eosinophilic material in the basement membrane</td>
<td></td>
</tr>
<tr>
<td>- Well-defined band-like zone of cellular infiltration that is confined to the superficial lamina propria, consisting mainly of lymphocytes</td>
<td></td>
</tr>
<tr>
<td>- Liquefaction degeneration in the basal cell layer</td>
<td></td>
</tr>
</tbody>
</table>

1.1.4 Epidemiology

OLP prevalence rate ranging from 0.1-4 % has been reported for different geographical locations.23, 44, 45 In England, the prevalence has been reported to be between 1-2.4 %.46 In India and Iran, it has been reported to vary between 0.4-2 %44, 47 while some East Asian studies showed prevalence’s of 2.15 % in
Malaysia, 3.85 % in Thailand, 1.0 % in Japan, and China 0.81 %. A computer-based literature search was conducted in Saudi Arabia and identified a prevalence of around 1.7, %\(^2\) while studies in Europe reported a prevalence of 0.6 % in Germany, 1.46 % in Italy, 1.27 % in Sweden, and 0.08 % in Hungary. A prevalence of 1.1 % has been reported in United Stated.\(^3\)

Huge variations in prevalence reported in some publications may be contributed to by geographical area, study background (clinical or population-based), and the lack of a standardised diagnostic methodology. Li C et al addresses the unintegrated information regarding global prevalence and incidence of OLP by performing a systematic review and meta-analysis. They concluded the overall estimated pooled prevalence as 0.89% among the general population and 0.98% among clinical patients. Furthermore, they also demonstrated higher prevalence in non-Asian countries, among women, and among people aged above 40 years.\(^4\)

1.1.5 Management and Prognosis

There remains no curative therapy for OLP and therefore management is aimed at reducing painful symptoms, which typically is achieved through healing of mucosal erosion and ulceration,\(^5\) shortening the length and severity of symptomatic outbreaks,\(^6\) and reducing the risk of developing oral cancer.\(^7\) Asymptomatic reticular and plaque forms do not require pharmacological treatment,\(^8\) however they still need to be closely monitored by a physician.
Table 1.2 Characteristic of 15 Population-Based Studies of Oral Lichen Planus (OLP) (Courtesy of Li C et al., 2020) (51)

<table>
<thead>
<tr>
<th>Source</th>
<th>Continent</th>
<th>No.</th>
<th>Prevalence</th>
<th>Age.y</th>
<th>Study Period</th>
<th>Diagnostic method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chher et al, 2018 (Cambodia)</td>
<td>Asia</td>
<td>28</td>
<td>1.71</td>
<td>≥18</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Mehrotra et al, 2017 (India)</td>
<td>Asia</td>
<td>81</td>
<td>0.02</td>
<td>≥15</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Feng et al, 2015 (China)</td>
<td>Asia</td>
<td>110</td>
<td>0.81</td>
<td>All ages</td>
<td>2012-2013</td>
<td>C</td>
</tr>
<tr>
<td>Carrad et al, 2011 (Brazil)</td>
<td>South America</td>
<td>14</td>
<td>0.088</td>
<td>14-104</td>
<td>2001</td>
<td>C(P)</td>
</tr>
<tr>
<td>Chung et al, 2005 (China)</td>
<td>Asia</td>
<td>1075</td>
<td>2.98</td>
<td>≥15</td>
<td>1998-1999</td>
<td>C</td>
</tr>
<tr>
<td>Espinanza et al, 2003 (Chile)</td>
<td>South America</td>
<td>19</td>
<td>2.14</td>
<td>≥65</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Jahanbani, 2003 (Iran)</td>
<td>Asia</td>
<td>6</td>
<td>0.51</td>
<td>18-69</td>
<td>NR</td>
<td>C+P</td>
</tr>
<tr>
<td>Reichart, 2000 (Germany)</td>
<td>Europe</td>
<td>21</td>
<td>1.04</td>
<td>35-44 for younger adults 65-74 for senior citizens</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Kovac-Kavcic and Skaleric, 2000 (Slovenia)</td>
<td>Europe</td>
<td>13</td>
<td>2.34</td>
<td>25-75</td>
<td>1983-1987</td>
<td>C</td>
</tr>
<tr>
<td>Zain et al, 1997 (Malaysia)</td>
<td>Asia</td>
<td>45</td>
<td>0.38</td>
<td>≥25</td>
<td>5 mo</td>
<td>C</td>
</tr>
<tr>
<td>Ikeda et al, 1995 (Cambodia)</td>
<td>Asia</td>
<td>24</td>
<td>1.82</td>
<td>15-99</td>
<td>1991</td>
<td>C</td>
</tr>
<tr>
<td>Vigild, 1987 (Denmark)</td>
<td>Europe</td>
<td>3</td>
<td>0.44</td>
<td>≥60</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Axel and Rundquist, 1987 (Sweden)</td>
<td>Europe</td>
<td>410</td>
<td>2.02</td>
<td>≥15</td>
<td>NR</td>
<td>C+P</td>
</tr>
<tr>
<td>Lay et al, 1982 (Burma)</td>
<td>Asia</td>
<td>23</td>
<td>0.38</td>
<td>≥15</td>
<td>NR</td>
<td>C</td>
</tr>
</tbody>
</table>

Abbreviations: C, clinical diagnosis, C(P), after clinical diagnosis, histopathological diagnostic procedures were performed if necessary; C+P, both clinical diagnostic and pathological diagnosis. NR, not reported.

1.1.5.1 Corticosteroid

Corticosteroids represent the current gold standard therapy for OLP, and are used topically in the vast majority of patients in treating mild to moderately symptomatic lesions. (52) Commonly prescribed topical corticosteroids are 0.05% clobetasol propionate gel, 0.1% or 0.05% betamethasone valerate gel, 0.05% fluocinonide gel, 0.05% clobetasol butyrate ointment or cream, and 0.1% triamcinolone acetonide ointment which represent decreasing potency respectively. (52) Betamethasone
Topical steroid application results in fewer disadvantages compared with systemic administration. However, there are still a number of adverse effects such as candidiasis, thinning of the oral mucosa and discomfort on application.\(^{(52)}\) Topical formulations of the more potent corticosteroids can cause adrenal suppression if used in large amounts for prolonged periods or applied with occlusive dressings. As a rule, the lowest-potency steroid that proves effective should be used.

In some instances, intralesional injection of corticosteroid for recalcitrant or extensive lesions involves the subcutaneous injection of 0.2–0.4 mL of a 10 mg/mL solution of triamcinolone acetonide by means of a 1.0-mL 23- or 25-gauge tuberculin syringe.\(^{(53, 54)}\)

Other topical immunosuppressants such as tacrolimus and pimecrolimus can also be used to induce remission of erosion/ulceration and reduce related painful symptoms.\(^{(25)}\) Systemic corticosteroids (prednisolone) and immunosuppressant (azathioprine or mycophenolate mofetil) can be used in the small group of patients who do not respond to topical therapy.\(^{(25, 26)}\) However, care must be taken to control side effects specifically for patients that using it for the long-term.\(^{(43, 55)}\) Other modalities treatment for OLP are calcineurin inhibitors such as ciclosporin A, tacrolimus and pimecrolimus. Those alternatives reported as caution due to some report on systemic adverse event.\(^{(56-58)}\)

### 1.1.5.2 Other modalities

Photo biodynamic therapy which uses low energy laser therapy has been used to treat OLP. It can trigger pain, discomfort, burning sensation, taste changes. NdYAG Laser and narrowband UV-B phototherapy have also been reported as an alternative approach for recalcitrant OLP cases.\(^{(59-62)}\)
Herbal medicine attracted some attention as an alternative therapy for OLP treatment.\(^{(63)}\) Most of the known health-benefits conveyed by herbal remedies result from antioxidants and anti-inflammatory chemicals within the mixtures, which hypothetically could ameliorate some of the symptoms of OLP.\(^{(63)}\) Some of the suggested herbal remedies are *aloe vera*, curcumin, *Glyrrhiza glabra*, pursiane, some Chines medicines, raspberry leaf extract, lycopene, and propolis which is a bee product.\(^{(59, 63-67)}\) Those herbal products suggested a single dose or as an adjunct to other therapies. In general, the above reported alternative therapy demonstrated range effect for OLP severity from no to significant benefit. Nevertheless, all review or study with alternative adjunct therapy demonstrated no or minimal adverse event with measured dose which might be worth as the main advantage for adjunct therapy.

OLP lesions usually healed within 6-12 months, but, some phenotypes such as the hypertrophic variant can persist for a long time even with application of topical steroid.\(^{(68)}\) In addition, to the long duration of the lesions, OLP is characterize by relapsing and recurrent episodes affecting either the same or different locations and with different degrees of severity.

WHO classified OLP as potential malignant disorder, which refers to the increased potential for carcinogenic transformation, with transformation rate of 1.1%. The changes were higher among smokers, alcoholics, and Hepatitis C Virus infected patients.\(^{(5, 69)}\) However, there are still questions as to whether the associated risk of malignant transformation is intrinsic to the OLP lesions or as a result of the patients’ immune response or genetic background.\(^{(68-70)}\)
1.2 Immunology background and its association with OLP

The body’s outer surface, which includes the oral cavity, acts as a protective barrier towards any challenge from the environment. Unlike the skin that is mechanically protected by several epithelial layers and up to 30 layers of dead cells, the surface of other mucosal organ such as the oral mucosal and the gastrointestinal, respiratory, and urogenital tracts are mostly covered with a single-layered epithelium. (71)

However, the interface between the organism and the outside world is also the site of the exchange of nutrients, and the export of products and waste components. It is mandatory for the oral mucosal to be selectively permeable; at the same time, facilitated with local defence mechanisms against environmental threats (e.g., invading pathogens). The mucosal immune system has evolved mechanisms for discriminating between harmless antigens from food and microflora and dangerous antigens and pathogens.

Characteristic features of mucosal immunity distinguishing it from systemic immunity are:(71)

1. Strongly developed mechanisms of innate defence
2. The existence of characteristic populations of unique types of lymphocytes and their products
3. Colonization of the mucosal and exocrine glands by cells originating from the mucosal-organized tissues (“common mucosal system”)
4. Transport of polymeric immunoglobulins through epithelial cells into secretions (sIgA); and
5. Preferential induction of inhibitory mechanisms directed against mucosal nondangerous antigens (“oral/mucosal tolerance”)
1.2.1 Innate immune system

The innate mucosal immune system in the oral cavity is composed of a number of cells (epithelial cells, macrophages, dendritic cells, mast cells, and other cells) and their humoral products (e.g., antimicrobial proteins, peptides and immunoglobulins). Basic functions of the mucosal immune system are protection against pathogenic microorganisms and prevention of penetration of immunogenic components from mucosal surfaces into the internal environment of the organism (barrier and anti-infectious functions). Another important function is the induction of unresponsiveness of systemic immunity to antigens present on mucosal surfaces (oral/mucosal tolerance) and the maintenance of homeostasis on mucosal surfaces (immunoregulatory function). Another important function is signalling communication to adaptive immune system to initiate complex immune system as a whole.\(^\text{71, 72}\)

1.2.1.1 Macrophages

Macrophages are tissue-resident phagocytic cells derived from blood monocytes, which are recruited into the tissues by chemokine signals such as monocyte chemotactic protein-1 (MCP-1).\(^\text{73}\) Macrophages have an array of important functions; they recognise and kill pathogens, initiate and resolve inflammation, and heal and prime the adaptive immune system leading to the survival of the host (Figure 1.3).\(^\text{74}\) During infection they can engulf pathogens by a process known as phagocytosis and can subsequently kill the pathogen through direct attack by reactive oxygen and nitrogen species, and non-oxidative mechanisms that include exclusion of nutrients, and lowered pH and digestive enzymes such as lysozymes. Macrophages also instruct cells of the adaptive
immune system to activate and prime the T cells by presenting fragments of the pathogen (in the presence of co-stimulatory molecules and differentiating cytokines), which initiates a memory response designed specifically for their clearance. Of equal importance is the role macrophages have in halting the immune response after pathogens have been cleared. They produce the anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), which dampen down an inflammatory episode by downregulation of pro-inflammatory cytokines and antigens, and through the induction of regulatory T cells that suppress antigen-presenting cells and T cell effector responses. They also aid tissue regeneration after inflammatory damage through the production of matrix metalloproteinases (MMPs) and their inhibitors, TIMPs (tissue inhibitors of metalloproteinases) that remodel tissue. This diverse array of macrophage effector functions has prompted the classification of distinct subsets along a phenotypic spectrum defined by their effector functions; pro-inflammatory or host defence (M1) to anti-inflammatory or regulatory (M2). M1 macrophages develop in an inflammatory setting; interferon gamma (IFN-γ) with microbial products— for example, lipopolysaccharide, or differentiation factors such as granulocyte macrophage colony-stimulating factor (GMCSF).

M1 macrophages have potent antimicrobial potential through the generation of reactive nitrogen species by the induction of inducible nitric oxide synthase and by increased production of reactive oxygen species. Conversely, macrophages exposed to an immunosuppressive or anti-inflammatory environment (IL-4 and IL-13 immune complexes; IL-10 macrophage colony stimulating factor (M-CSF) or glucocorticoids, or both) adopt an anti-inflammatory or regulatory M2 phenotype. They are predominantly anti-inflammatory through
their secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-1 receptor antagonist (IL-1Ra), and are characterised as IL-12LOW, IL-23LOW, and IL-10HIGH.

M2 macrophages have expressed lower antimicrobial activity than M1 macrophages, but express higher levels of scavenger receptors such as mannose receptors, which correlates with their role in tissue repair, homeostasis, and clearance of cell debris. To induce a state of tolerance the balance of macrophage subsets in the lamina propria of healthy oral mucosa is likely to be tipped in favour of an M2 phenotype. As disruption to this balance can lead to an inappropriate or exaggerated response to particular stimuli, macrophages can potentially aid the progression of inflammatory and immunosuppressive oral diseases, and are therefore promising candidates for cell-based therapeutic targets. (73)

OLP could be exacerbated by M1 phenotype. (76) Infiltrating monocytes recruited into the lesion develop a pro-inflammatory M1 phenotype because of the high levels of GM-CSF, TNF-α, and IFN-γ produced at the site. (6, 77) There were three main mechanism contributed to the OLP lesion severity ie initiation of inflammation through release of pro-inflammatory cytokines which leading to upregulation of cell adhesion molecules on endothelial and keratinocyte surfaces and induce chemokine expression by oral keratinocytes, (76, 78) activation and priming of T cells, (79) and direct destruction of the basal membrane.
At the site, macrophages can activate antigen-specific T cells and directly influences the polarisation of T cells through the secretion of differentiation cytokines (IL-12→Th1 or IL-4, IL-5→Th2, and IL-17A→Th17).\(^{(80, 81)}\) T cells in the disease have been found to secrete IFN-\(\gamma\), which is typical of Th1 subsets, and is indicative of IL-12 production by the macrophages in oral lichen planus. IFN-\(\gamma\) and IL-2 are cytokines produced by activated Th1 cells, and they function to permit the full activation of CD8+ cytotoxic T cells, which are hypothesised to kill basal keratinocytes.\(^{(82)}\) IFN-\(\gamma\) can also feedback and activate the M1 macrophages to produce TNF-\(\alpha\) which can directly initiate basal keratinocyte apoptosis, and indirectly increase the rate of destruction of the basal membrane through the upregulation of MMP-9 from lesional T cells.\(^{(82, 83)}\) MMP-9 cleaves type IV collagen causing the membrane to be destroyed and the subsequent loss of attachment of basal keratinocytes, which potentially results in keratinocyte apoptosis and further

**Figure 1.3 Functionally diverse of Macrophages.** M1 has more inflammatory setting with potent antimicrobial potential through reactive nitrogen species. M2 express lower antimicrobial activity but express higher levels of scavenger receptors. Adapted from Merry R et al, 2012.
damage.\(^{(64)}\) Macrophages are distributed close to the damaged basal layer and can therefore contribute to the destruction of the basal membrane. There seems to be a vicious cycle of perpetuating inflammation and damage to the basal membrane, as this destruction can further initiate inflammation through the release of damage-associated molecular patterns (DAMPs).\(^{(31)}\) M1 macrophages can therefore aid the progression of lichen planus by activating T cells and exacerbating inflammation at the site.\(^{(76)}\)

### 1.2.1.2 Epithelial cells

Oral epithelial cells carry a burden responsibility to protect the inner part of the mucosa from microorganisms, as well as maintaining their integrity. Marshall, demonstrated that oral keratinocytes cells had the potential to induce both CXCL9 and CXCL10 expression under IFN-γ stimulation. Both chemokines are absent or only produced to a minimal degree in the healthy oral mucosa. Interestingly, this study also showed higher CXCL9 and CXCL10 expression from OLP keratinocytes in vitro compared to HC.\(^{(78)}\) CXCL9 and CXCL10 suggested as a key factors that stimulates T-cell migration into OLP lesions leading to the characteristic band-like accumulation of lymphocyte within the lamina propria, overlying keratinization and liquefactive degeneration of basal keratinocytes.\(^{(8)}\)

Although oral epithelial cells are not immune cells, they are capable of mounting an immune response. Oral epithelial cells express toll-like receptors (TLR), which are a family of pattern recognition receptors (PPRs) that perform functions in both the innate and adaptive immune responses.\(^{(85)}\) TLRs can be found on the cell surface (1, 2, 4, 5, 6 and 10) and are able to recognize external pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharides (LPS), peptidoglycans and lipoproteins. Those PAMPs along
with other cytokines such as IFN-γ will lead to the expression of IFN-inducible
genes and drive polarization, activates signal transducer and activator of
transcription (STAT) pathway. Once the STATs are activated, it induces the
transcription of suppressor of cytokine signalling 1 (SOCS1) (Figure 1.4). TLRs
(3, 7, 8, 9) can also be located within intracellular vesicles (endosomes) and these
predominantly recognize microbial nucleic acids. Some TLRs are also capable
of mediating responses to host molecules, such as heat shock proteins (HSP), β-
defensins and extracellular matrix degradation products that occur in injured or
inflamed tissues. Activation of TLRs with their specific ligands initiates an immune
response and the activation of a host of transcription factors including nuclear
factor (NF-κB), leading to the production of antimicrobial immune mediators.

Defects in TLR activation have been associated with numerous diseases
and may contributed to the dysfunctional innate immune response in oral lichen
planus. Ohno, reported increased expression of TLR1, 2, 3, 4, 6 and 10 in OLP
tissues compare to normal mucosa. This supported by another study, which
demonstrated increased soluble TLR4 in saliva of OLP patients.

Another important role by epithelial cell is the maintenance of membrane
integrity, which has been shown to be defective and contributed to OLP
pathogenesis. The physical barrier function of the epithelium mainly depends
on tight junctions, which include adhesion molecule (JAM)-A, occludin, claudins,
zonula occludens (ZO)-1, and ZO-2. Other form of cellular junction is the adherent
junctions (AJs) which consist of E-cadherin, nectins, and β-catenin. Defects in
these cellular junctions can contribute to the development of tissue and systemic
inflammation.
Figure 1.4. The JAK/STAT pathway is involved in the miR155/SOCS1 axis in the immune system. When immune cells are stimulated by cytokines, including IFN-γ and LPS, the JAK/STAT pathway is activated. Phosphorylated STATs form a complex and are translocated to the nucleus, where they activate or repress the transcription of target genes. In macrophages, this pathway induces M1 polarization and promotes the production of tumor necrosis factor-α, IL-6 and IL-1β. JAK/STAT signaling also regulates IL-12 production and DC maturation. Furthermore, the JAK-STAT pathway contributes to Treg and Th17 differentiation and regulates the competitive ability and proliferative potential of Tregs. SOCS1 negatively regulates the JAK/STAT pathway by binding to phosphorylated JAKs. miR-155 targets SOCS1 and inhibits its expression, leading to decreased binding of SOCS1 to phosphorylated JAKs. Therefore, miR-155 positively regulates the JAK/STAT pathway. JAK, Janus kinase; STAT, signal transducer and activator of transcription; IFN, interferon; LPS, lipopolysaccharide; miR, microRNA; SOCs, suppressor of cytokine signaling; Th, T-helper cell; Treg, T-regulatory cell; IL, interleukin; rbx, ring box; DC, dendritic cell. (Adapted from Tao Y, et al, 2019).
1.2.2 Adaptive Immune function

Adaptive immune is the second more sophisticated line of body’s defence which activated by a signal from the innate immune. The function is to destroy invading pathogens and any toxic molecules they produce. Its function mainly based on the highly specific response to the particular pathogen that induced them. Equipped with T-cell memory, protection they offer can be long-lasting.\(^\text{(89)}\)

Adaptive immune or acquired immune response is mediated through the generation of antigen-specific lymphocytes that are capable of recognising a vast array of antigens either directly or via antigen presentation by the major histocompatibility complexes (MHC). The process of generating antigen specific adaptive immunity occurs in the secondary lymphoid organs, such as, lymph nodes, spleen, Peyer's patches, and mucosal tissues such as the nasal-associated lymphoid tissue, adenoids, and tonsils.\(^\text{(72)}\)

Langerhans cells (LC), which are professional antigen presenting cells, have been reported to be present in higher numbers in patients with OLP.\(^\text{(90-92)}\) The increase in Langerhans cells in OLP suggests a possible significant role of the adaptive immune response in OLP pathogenesis. In a study assessing biopsy samples from 30 OLP and 35 oral squamous cell carcinoma (OSCC) and 30 Healthy control (HC) by immunohistochemistry (IHC) using anti S100 antibody for LC detections. Both OLP and OSCC group demonstrated significant change in mean value compared to HC.\(^\text{(91)}\) On another study, 18 OLP and 10 normal mucosa biopsies were immunostained for CD4, CD8, CD1a, leucocyte function-associated antigen 1 (LFA-1), vascular adhesion molecule-1 (VCAM-1), ligand intercellular adhesion molecule-1 (ICAM-1). The result demonstrated CD4+ and CD8+ cells expressed higher LFA-1, while both ICAM1 and VCAM-1 were significantly higher
in OLP patients compared to control patients.\(^{93}\) The authors hypothesised that in OLP, there may be an immune response to some unknown antigen within the basal keratinocytes and activated LCs present the antigen to CD4+ cells that, through adhesion molecules, promotes CD8+ cell induced epithelial destruction.

Moreover, an assessment of Forkhead box protein 3 (FoxP3+) cell subsets between different subtypes of OLP found that atrophic OLP showed the highest number of FoxP3+CD4+ T cells with the ulcerative form of OLP the lowest. Interestingly, many of the observed FoxP3+CD4+ T cells expressed T-bet, an IFN-γ hallmark transcription factor, suggesting these cells have an inherent capacity to enhance rather than suppress inflammation, a factor that could explain the chronicity of OLP.\(^{94}\)

IL-25 (also known as IL-17E) and IL-4 mRNA levels have been reported to be increased and correlate significantly with each other in specific OLP subtypes compared to HC. \(^{95}\) These result came from 13 reticular OLP, 12 erosive OLP, and 12 HCs specimen tissues. Further investigation with recombinant IL-25 in vitro demonstrated an ability to significantly increase the numbers of CD4+T cells from both subtypes of OLP and elevate the production of IL-4 mRNA. The increase in IL-25 may be related to the elevated expression of IL-25R identified in local OLP lesions. Statistical analyses demonstrated that the simultaneously increased levels of IL-4, CXCL8 and CCL20 in keratinocytes were induced by IL-25, but not by the closely related IL-17 A or IL-17 F. Decreasing IL-25R subunit expression by siRNA mediated knockdown significantly blocked the expression of all cytokine-produced inflammatory mediators in oral keratinocytes. The author suggested that in OLP lesions, IL-25 can function to mediate a Th2 response, which may be important cause of chronicity and persistent inflammation.\(^{95}\)
1.3 Oral lichen planus association with oral dysbiosis

The Oral microbiome is defined as collective communities of bacteria that live in the oral cavity. It is suggested that the oral microbiome plays an important role in some pathogenic conditions such as dental caries, periodontal disease, osteonecrosis and halitosis. It also been implicated as a reservoir for infection at other body sites, and in the pathogenesis of non-oral diseases such as diabetes mellitus, metabolic and pulmonary diseases, rheumatoid arthritis, stroke and cardiovascular diseases.

In OLP, oral microbiome imbalance, termed dysbiosis, has been suggested as:

1. Potential etiologic factor
2. Contributing factor for the clinical manifestation changes over time
3. Causative factor for the periods of exacerbation

Choi, investigated the intracellular bacteria within the oral epithelia tissue through the use of in situ hybridization using a universal probe targeting the bacterial 16S rRNA gene in combination with immunohistochemistry with anti-CD3, anti-CD4, anti-CD8, and anti-macrophage-specific antibodies. The study demonstrated that bacteria were abundant throughout the epithelium and the lamina propria of OLP tissues, which exhibited positive correlations with the levels of infiltrated CD3+, CD4+, and CD8+ cells. Interestingly, this group also detected bacteria presence within the infiltrated T cells. Pyrosequencing analysis of the mucosal microbiota from 13 OLP patients and 11 control subjects was in agreement with other periodontal disease demonstrated a decrease in Streptococcus and increases in gingivitis/periodontitis-associated bacteria in OLP lesions. This dysbiosis might be one reason for the frequent involvement of
gingival tissue in OLP lesions. Furthermore, Choi used isolated bacterial species of *Capnocytophaga gingivalis* and *Streptococcus sanguinis* to demonstrated that certain oral bacteria can damage the epithelial physical barrier and are internalized into epithelial cells or T cells, resulting in the production of T cell chemokines CXCL10 and CCL5.\(^{100}\)

Wang, reported that the overall structure of the salivary microbiome was not significantly affected by disease status.\(^{101}\) However, they did observe variations in abundance for several taxonomic groups in OLP. *Porphyromonas* and *Solobacterium* showed significantly higher relative abundances, whereas *Haemophilus*, *Corynebacterium*, *Cellulosimicrobium* and *Campylobacter* showed lower abundances in OLP patients, as compared with HC. Furthermore, this group reported the disease severity and immune dysregulation were also genus associated, with *Porphyromonas* correlating with disease scores and salivary levels of IL-17 and IL-23.\(^{101,102}\)

Recently, Yan *et al* reported higher level of bacteria compare to fungi population in OLP salivary microbiome. Between those fungi, *Candida* and *Aspergillus* genus demonstrated significantly higher abundances within reticular OLP while *Alternaria* and *Sclerotiniaceae_unidentified* in erosive OLP. Specifically, *Aspergillus* recognized as an ‘OLP-associated’ fungus due to their higher frequency compared to the HC. The author subsequently concluded that fungal dysbiosis could alter the salivary bacteriome, may reflect a direct effect of host immunity, or secondary contribution to steroid application which contribute in OLP pathogenesis.\(^{103,104}\)
1.4. Potential role for probiotics in oral lichen planus treatment

1.4.1 Probiotic definition

Probiotic is defined as a live microorganism that purposely confers a health benefit on the host. For over 2,000 years, these agents have been used to treat diarrhoea and since that time extensive research been conducted to prove the evidence to support the benefit of certain bacteria to human health. Pubmed has indices more than 20,000 research articles studying probiotics. Eli Metchnikoff, a Professor at the Pasteur Institute in Paris and Nobel Laureate of Medicine and Physiology first reported the beneficial health properties of yogurt containing Lactobacillus bulgaricus in 1908. Probiotics are emerging as a potential viable alternative for immunotherapy either as a single dose or as adjunctive in regulating inflammation cytokines in some diseases.

1.4.1 VSL#3

One of the most commonly used probiotic is VSL#3, which is a lyophilized mixture consisting of eight different Gram-positive organisms (Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Streptococcus salivarius subsp. thermophilus). It is marketed as the most concentrated probiotic currently available over the counter in the UK. It is registered as a food supplement and has a BNF (British National Formulary) indication for the maintenance of remission of ileoanal pouchitis in adults. It is also available over the counter and distributed by Ferring Pharmaceutical Ltd.
1.4.3 Use of probiotic for treatment

VSL#3 has been studied clinically and experimentally and reported to modulate the innate immune system and cytokine secretion in inflammatory bowel disease (IBD). \(^{105-110}\) IBD is composed of two main idiopathic pathologies, Crohn’s disease (CD) and ulcerative colitis (UC) with an extraintestinal involvement within the oral cavity \(^{111-113}\) Indurated tag-like lesions, cobblestoning, and muco-gingivitis are the most common specific oral findings encountered in CD cases. Additionally, Aphthous stomatitis and pyogenic stomatitis vegetans are among non-specific oral manifestation of IBD. \(^{113}\)

A Meta–analysis conducted by Mardini using five studies consisting of 144 UC subjects who consumed VSL#3 demonstrated reduced symptoms in 75% of patients compared with only 25% in the placebo cohort. \(^{114}\) In addition to effects in IBD, Wong et al reported a beneficial effect of VSL3#3 in irritable bowel syndrome with significance improvement in abdominal pain, distention intensity, increased release of salivary morning melatonin, increase in satisfaction with bowel movements and quality of life. \(^{115}\) A study by Mariman reported that this agent interfered with chemokine expression and secretion in human dendritic cells, exerting an anti-inflammatory effect. \(^{116, 117}\) More recently, Wang, investigated the effect of probiotic VSL#3 on NF-κB and TNF-α in three parallel arms of 60 Sprague Dawley rats with colitis and reported that expression of TLR4 and NF-κB, and the levels of NF-κB, TLR4, and TNF-α were decreased. Additionally, this group reported positive correlation between TLR4 and NF-κB, and suggested the VSL#3 to be a first choice of drug for colitis. \(^{118}\)

A potential mechanism of action of VSL#3 on cytokine secretion was described by Hormansprenger et al and involved the blockade of intracellular
cytokine trafficking. They reported that VSL#3 could directly inhibit CXCL10 secretion. Furthermore, an elevation in systemic and local CXCL10 levels has been directly implicated in a number of diseases but significantly it is strongly associated with UC and is even a therapeutic target currently undergoing clinical trials. (111, 119) For CD, VSL#3 was also reported to confer a modest protective effect resulting in disease recurrence, and reduced CD activity. (120-122) Based on these and other probiotic research an expert panel of International Scientific Association for Probiotics and Prebiotics (ISAPP) held in October 2013 concluded that most strains of these species were expected to trigger a generic or core effect on the guts physiology supporting a healthy immune system. The meaning of supporting a healthy immune system included preventing allergic disease, down regulation of inflammation, and enhancement of anti-infection activities. (108)

1.4.4 Use of probiotic for oral mucosal disease

Overall studies suggest that the probiotic VSL#3 is a potent regulator of inflammation capable of changing or controlling the inflammatory cytokines within the mucosal epithelium. We also know that the oral mucosa can be influenced by probiotics as two previous reports used probiotics in Behcet’s disease and recurrent aphthous stomatitis (RAS), with the authors describing a reduction in the number of oral ulcerations and subjective relief of oral discomfort. (123, 124)

More recently, Jiang performed a randomized, double-blind, placebo-controlled trial using probiotic mixtures of Bifidobacterium longum Lactobacillus lactis, and Enterococcus faecium on oral mucositis induced by chemotherapy for patients with nasopharyngeal carcinoma and reported a significant reduction in the
severity of oral mucositis.\textsuperscript{(125)} This study was in agreement with another study, that used the probiotic \textit{Lactococcus lactis} as a mouth rinse for oral mucositis.\textsuperscript{(126)}

In the case of RAS treatment, Pedersen, employed a double-blind randomized, placebo-controlled design with two parallel arms. The intervention consisted of lozenges containing two strains of \textit{Lactobacillus reuteri} taken twice daily for 90 days. An improvement of the ulcer severity score as well as oral pain, was evident in both active and placebo groups after 90 days but the reduction was only statistically significant compared with baseline in the probiotic group. There were no significant differences between the groups, neither at baseline nor at follow-up. No side effects were recorded. Daily supplements with \textit{L. reuteri} reduced the severity of aphthous lesions over a 90-day period but the improvement was not significantly better than placebo.\textsuperscript{(127)}

\textbf{1.4.5 Potential of VSL\#3 probiotic for oral lichen planus}

To date none of the VSL\#3 studies reported have involved OLP patients even though there are strong similarities between intestinal gut and oral mucosa. Oral mucosal epithelium is the first part of the gastrointestinal tract. The oral epithelial surface shared the same function of mucosal association of lymphoid tissue (MALT) with a specific immune organization protecting nearly the entire inner surface of the human body from the oral-pharyngeal cavity, gastrointestinal tract, respiratory tract, urogenital tract and exocrine glands.\textsuperscript{(128, 129)} The resemblance of oral-pharyngeal immune compartment with the intestinal tract epithelial is the representative of the MALT organs that are buccal mucosa, salivary glands, and Waldeyer’s ring and the presence of inductive and effector sites in the epithelial layer. The other similarities are the compartmentalized immune cells
such as the intra-epithelial lymphocytes, epithelial cells and lamina propria DC and macrophages which are capable of the elimination of foreign antigens.\(^{(128)}\)

Aetiology of OLP is poorly understood, but classified as an autoimmune disease with dysfunctional innate immune response by TLR activation.\(^{(31, 48, 68, 130-134)}\) Dysfunctional innate immune responses have also been identified in other chronic mucocutaneous diseases such as CD and UC.\(^{(135-142)}\) Katsanos stated that IBD patient has an increased risk for both oral cancerous and malignant transformation of precancerous lesions.\(^{(143)}\)

TLRs signalling, either in the gastrointestinal tract (GI) or oral mucosa, results in the release of inflammatory cytokines and chemokine that have the potential to contribute to disease activity and severity.\(^{(144, 145)}\) A number of cytokines have been shown to contribute to OLP, these include CCL5/CCR5, IL-2, IL-8, TNF-\(\alpha\), IFN-\(\gamma\), TGF-\(\beta1\), IL-12, IL-14, IL-10, IL-6, GM-CSF, IL-1\(\beta\), CXCL9, CXCL10, and CXCL11.\(^{(5, 12, 31, 43, 84, 132-134, 146, 147)}\) The current gold standard treatment for OLP is corticosteroids, which are known to dampen inflammation and cytokine secretion, however long term corticosteroid usage can result in adverse side-effects which are clearly problematic for the patient and clinician. In clinical terms, there are about 90 papers published to prove the efficacy of VSL#3 in a variety of gastrointestinal diseases. The studies demonstrate that VSL#3 is safe and effective in increasing remission rates in mild to moderately active UC and that the probiotic is a potent regulator of inflammation capable of changing or controlling the inflammatory cytokines within the mucosal epithelium. We also know that oral mucosal inflammation can be influenced by probiotic usage, as some published studies relevant to immunologically-mediated oral ulceration of RAS and Bechet's syndrome reported beneficial effects of probiotic in reducing oral ulcerations.\(^{(123, \ldots)}\)
We would therefore suggest that current data support the hypothesis that the use of VSL#3 may benefit OLP patients through the targeting of the inflammatory response within the oral mucosa.

1.5 Summary of investigations conducted and hypothesis

In this thesis, the probiotic VSL#3 effects on the oral epithelial innate immune response is characterized in vitro and in vivo.

In vitro: This study aimed to determine the immune modulator effects of VSL#3 and the individual bacterial species on oral epithelial cells.

In vivo: In clinical setting, OLP cytokines profiling was performed to characterise the cytokines profiles in OLP and compare the different phenotypes. Additionally, we will conduct the first in human clinical trial of a probiotic in OLP using a randomized, double-blind controlled feasibility trial of VSL#3.
CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 VSL#3 Isolation and generation of single species and verification

2.2.1 VSL#3 stock preparation for cells stimulation

VSL#3 (Ferring Pharmaceuticals, UK) was re-suspended in 50 ml phosphate buffered saline (PBS) (Gibco, UK) and centrifuge at 2000 g for 10 minutes. The pellet was resuspended in fresh 50 ml PBS and centrifuged at 2000 g for 10 minutes. This process was repeated a further two times. After the final wash the VSL#3 was diluted in PBS to a final stock concentration of $1 \times 10^{10}$ per ml. VSL#3 was then split in to three equal aliquots and either heat killed, formaldehyde fixed, or left viable. Heat killed protocol was perform at 90°C for 30 minutes and formaldehyde fixing was performed using 5% formaldehyde (Sigma-Aldrich, UK) for 3 hours at 4°C. Fixed bacteria washed three times in PBS. Killing efficiency was verified by plating 10 µl on Brain Hearth Infusion (BHI) agar (Sigma UK, 70138-500G) for three days at 37°C. All stock kept in -20°C until needed.

2.2.2 Single species isolation and identification

For single species isolation a number of different conditions were used to optimise the chances of obtaining the eight different species listed on VSL#3 information package. Since media and cultured condition were not described, then the growth conditions for each species were designed based on the common knowledge of individual strains. 10 µl of live VSL#3 stock ($10^{10}$ cfu/ml) was grown on BHI agar plates and incubated at either 37°C and 42°C for 24 hours. Isolation of bifidobacterum species, Bifido selective medium (BSM) was used (Sigma, Cat
no 88517 for agar and 90273 for broth) and incubated anaerobically for 3 to 5 days at 37°C.

In all cases, individual colonies were picked based on different visual morphology, size, and form, and elevation, margin of the whole colony, colour, surface texture, density, and consistency of the colony (Figure 2.1).

Individual colonies were then grown in BHI/BSM broth overnight followed by plating on a BHI/BSM agar plate for 24 hours. Each single colony isolate was then identified using Direct PCR (Polymerase chain reaction) of intact bacteria (colony PCR) method. One single colony of each isolate was added to 15 µl PCR master mix along with a complete, ready-to-use, 2x Biomix containing ultra-stable Taq DNA polymerase (Bioline USA, cat no.BIO-25012), 1 µl of each primer forward and revers of CGGTTACCTTGTTACGACTT and AGAGTTTGATCMTGGCTCAG, 13 µl distilled H₂O. The thermocycling program of 94°C for 10 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute 30 second, followed by 75°C for 10 minutes. The PCR product was checked using agarose gel electrophoresis. A 1% agarose (Bioline USA, cat no. BIO-41025) in Tris-buffered saline (TBS) pH 7.5 was heated to 95°C and cast into a gel tray with an appropriate comb. 10 µl of each PCR reaction was mixed with GelRed® (Biomix, cat no 41003-1) 2 µl as intercalator and loaded into individual wells and 1 µl of a 100 bp DNA Ladder (Invitrogen, Cat no. 15628-019) was added as a reference. The Agarose gel was run at 80 Amps/Volts for 30 mins. Gels were imaged using a Thermo Scientific MYECL imager.

The rest of the PCR product then purified Using QIA quick purification kit (QIAGEN, Cat No. 28104), then 50 ng/ml of the purified product along with 10 µl 16S reverse and forward primers send for sequencing to Beckman Coulter.
Genomics (BeckmanGenomics.com). The sequencing result from Beckman Coulter got back in zip form files with each sequencing contain SEQ and AB1 files code. After files extraction, the AB1 extension file used to identify the sequencing result using Biotools sequencing editor and compare via BLAST database library at http://blast.ncbi.nlm.nih.gov.

<table>
<thead>
<tr>
<th>Form</th>
<th>punctiform</th>
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<tbody>
<tr>
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<td>circular</td>
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<td>undulate</td>
<td>filamentous</td>
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<tr>
<td></td>
<td>lobate</td>
<td>curled</td>
</tr>
</tbody>
</table>

Figure 2.1. Different morphologically of the VSL#3 culture chosen based on illustration variety form. Image form and shapes. Procop et al (2017)

2. 2 Cell culture

2.2.1 THP-1 cell line

THP-1 (ATCC®TIB-202™) orign from eukaemia monocytic cell. These cells are commonly used to investigate macrophage/monocyte functions. THP-1 cells are routinely maintained in Falcon tissue culture flasks 750 ml (CORNING) containing RPMI-1640 medium GlutaMAX™ (Gibco 61870) supplemented with Fetal Bovine Serum (FBS) 10% (Sigma F9665), 20 mM HEPES (Sigma H0887),
100 U/ml Penicillin/Streptomycin (Gibco 15140-122), 50 mM 2-mercaptoethanol (Gibco 31350) and grown in 5% CO₂ at 37°C. Cells were maintained at a concentration of 2x10⁵ – 10⁶ cells/ml. THP-1 cells were split 1:1 with fresh media every two days and kept no longer than 3 months. Various number of cells seeded based on plate sized listed on table 2.1.

Table 2.1 List number of THP-1 and MOE1a cells seeded based on various plate sizes

<table>
<thead>
<tr>
<th>Plate</th>
<th>Number of cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 wells</td>
<td>10⁵</td>
</tr>
<tr>
<td>24 wells</td>
<td>3.5 x 10⁵</td>
</tr>
<tr>
<td>12 wells</td>
<td>5 x 10⁵</td>
</tr>
<tr>
<td>6 wells</td>
<td>10⁶</td>
</tr>
<tr>
<td>100 mm petri-dish</td>
<td>5 x 10⁶</td>
</tr>
</tbody>
</table>

2.2.2 MOE1a cell line

Mouth ordinary epithelium (MOE_1a) is a gingival epithelium cell line derived from a 28-year old human and provided to the lab by Prof Shosei Kishida of Biochemical Genetic Department of Kagoshima University Graduate School Medicine Dentistry. Some genes in this cell lines were transduced by CDK4R24C, cyclin D1, and hTERT with pS3C234.\(^{(152)}\) The cell line is maintained in the following media, Defined Keratinocyte-SFM (Invitrogen 10744) supplemented with 100 U/ml Penicillin/Streptomycin (Gibco 15140-122), and 0.2% bovine pituitary extract (BPE) and Epidermal Growth Factor (EGF) and grown in 5% CO₂ at 37°C. Cells were seeded at 1-2 x10⁵ per 75, ml flask and were 80% confluent after 7 days. To passage the monolayer, the cells were washed twice with sterile PBS and incubated for 5 minutes in 5% CO₂ at 37°C condition with 5 ml 0.12 % trypsin and 0.02 % EDTA (Ethylenediaminetetraacetic acid, Sigma, 59430C) in PBS to dissociated cell from the flask bottom. The monolayer was then scraped,
transferred to a 15 ml Falcon tube before being centrifuged at 1200 g for 10 minutes. Cells were washed 2 times in sterile PBS and resuspended in growth media (as above).

### 2.3 Viability assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a colorimetric metabolic activity indicator used in cell viability assays. The 5 mg/mL MTT buffer was made by dissolving 500 mg of MTT (Sigma M2128) in 100 mL of PBS. Cells were cultured in 96-wells plate within 100 μL of the medium under different conditions and time frames depending on the experimental protocol. The viability of the cells at the end of the protocol was determined by adding 30% of total volume of MTT lysis solution (30 μL) to the wells and incubating in 5% CO2 at 37°C condition for at least 4 hours. MTT is converted to formazan, a dark blue water-insoluble through the reducing activity of mitochondrial dehydrogenases in live cells. The plate are then centrifuged at 8000 rpm for 5 minutes and supernatant carefully removed. Formazan is solubilized through the addition of 100 ul lysis solution (900 mL isopropanol, 47 ml dH2O, 50 ml 10% sodium dodecyl sulphate (SDS) and 3 mL concentrated HCl). The solution is pipetted multiple times until the formazan crystals dissolve. Plates were read on a FLUOstar Omega microplate reader (BMG LABTECH) at OD563 nm.

### 2.4 Cytokines quantification

#### 2.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Cells supernatant from THP-1, MOE1a cells or human serum were tested for levels of CXCL10, IL-6, CXCL8, TNF-α using Duo-Set ELISA kits according to manufactures instructions (R&D) and IFN-γ by Biolegend max deluxe ELISA kit.
Briefly, capture antibody in PBS was incubate on a 96 well nunc immune polysorp plate (Nunc™ thermofisher, Cat No. 44-2404-21) overnight at room temperature (RT). The plate was washed in 0.01 % Tween-20 in PBS (wash buffer) and blocked using 1 % BSA in filtered PBS, for 1 hour at RT. The plate was washed three time in wash buffer and the cell culture supernatants and standards made up in reagent diluent (0.1 % BSA in PBS) added for 2 hours at RT. The plate was washed three times in wash buffer and the detection antibody in reagent diluent added for 2 hours at RT. The plate then washed three times with wash buffer and Horseradish-peroxidase streptavidin (HRP) in reagent diluent added 20 minutes at RT. The plate then washed three times with wash buffer and develop using tetramethylbenzidine approximately 20 minutes at RT and reaction stopped by the addition of 2 N H₂SO₄. The plates were read at an absorbance of 450 nm and 570 nm as a correction wavelenght for the polystyrene plate using a CLARIOstar (BMG technology).

**Table 2.2** List of ELISA kits used catalogue number, supplier and supernatant dilutions

<table>
<thead>
<tr>
<th>Kit</th>
<th>Catalogue #</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>THP-1</th>
<th>MOE1a</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL8/CXCL8 DuoSet ELISA</td>
<td>DY208</td>
<td>R&amp;D Systems</td>
<td>1 : 20</td>
<td>1 : 5</td>
<td>Neat</td>
<td></td>
</tr>
<tr>
<td>Human TNF-α DuoSet ELISA</td>
<td>DY210</td>
<td>R&amp;D Systems</td>
<td>1 : 10</td>
<td>1 : 5</td>
<td>Neat</td>
<td></td>
</tr>
<tr>
<td>Human IP-10 DuoSet ELISA</td>
<td>DY266</td>
<td>R&amp;D Systems</td>
<td>1 : 10</td>
<td>1 : 5</td>
<td>Neat</td>
<td></td>
</tr>
<tr>
<td>Human IL-1B DuoSet ELISA</td>
<td>DY201</td>
<td>R&amp;D Systems</td>
<td>1 : 10</td>
<td>1 : 5</td>
<td>Neat</td>
<td></td>
</tr>
<tr>
<td>Human IL-6 DuoSet ELISA</td>
<td>DY206</td>
<td>R&amp;D Systems</td>
<td>1 : 10</td>
<td>1 : 5</td>
<td>Neat</td>
<td></td>
</tr>
<tr>
<td>Human IFN-γ DuoSet ELISA</td>
<td>437004</td>
<td>Biologend</td>
<td>Neat</td>
<td>Neat</td>
<td>Neat</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Catalogue #, catalogue number
2.4.2 MSD Multi-Spot Assay System

To accurately measure cytokine levels in the serum of participants in the CABRIO study (Chapter 6) we used the Meso Scale Discovery (MSD) V-PLEX human proinflammatory panel I (MSD K15052D-1) which measure 4 cytokines IFN-α, IL-1B, IL-6, and TNF-α. Briefly, the MSD-96 well plate contain 4-spots pre-coated with capture antibody which were washed three times with 300 μl wash buffer (0.01% Tween-20 in PBS). Fifty microliters of serum diluted 1:1 with reagent buffer and cytokine standards were added and incubate at for 2 hours at RT, then washed x3. Detection antibodies conjugated with electrochemiluminescent (ECL) labels (MSD SULFO-TAG contain in the kit) added and incubate for 2 hours then washed x3. Read buffer T that creates the appropriate chemical environment for ECL then added before reading the plate on a SECTOR® Imager 6000 (MSD).

2.5 Gene Expression

2.5.1 RNA Purification

Total RNA was extracted and purified from THP-1 or MOE1a cells using RNeasy mini kits (Qiagen, Cat. No. 74104). Cells were harvested by centrifugation (500 g for 5 mins), and the supernatant was removed (or collected) and 350 μL of RLT lysis buffer (Qiagen, Cat No. 79216) with 1% beta-2- mercaptoethanol (M3148 Sigma) added and stored at -80°C prior to extraction. RNA was extracted using the RNeasy Mini Kit columns with RNase-free DNase treatment (Qiagen) and processed according to the manufacturer’s instructions with a RNase-free DNase treatment (Qiagen) Step. The concentration of total RNA in RNase-free H₂O (Qiagen) was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and/or Qubit 2.0 Fluorometer (Invitrogen). Ratio 260/280 around 2.0 considered as pure, we accepted 1.8 as the lowest RNA ratio for our experiment.
2.5.2 Complementary DNA (cDNA) synthesis

Total RNA was converted to cDNA using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) kit (M1705 Promega). 500 ng of total RNA diluted in 15 μL of RNase-free water was added to 1 μL of oligo dT primer (Sigma) and incubated for 10 minutes at 70 °C for primer annealing and then placed on ice for 5 minutes. After that, a master mix containing 1 μL of M-MLV RT, 5 μL of 5X Reaction Buffer, 1 μL Recombinant RNasin® Ribonuclease Inhibitor (Promega), and 2 μL of dNTP Mix (NU-0010-10 Eurogentec) was added to each sample making a total volume of 25 μL. Samples were incubated at 40 °C for 10 minutes for DNA polymerization and heated at 70 °C for 5 minutes for enzyme deactivation on a DNA Engine Tetrad 2® Peltier Thermal Cycler (Bio-Rad). Samples were made up to 100 μL with RNase-free water and stored at -20 °C.

2.5.3 DNA Purification

Total DNA was extracted using Bacterial genomic DNA purification kit (Edge Bio, 85171) according to manufacturer instructions. Briefly, 2 ml of participant’s saliva prepared samples (containing 2 ml saliva buffer composed reagents listed on table 2.3 and 2-5 ml saliva) or 2 ml of bacterial overnight or time frame cultured was centrifuged for 10 minutes at 3000 g. The supernatant then removed (or collected) and added with 400 μl of Spheroplast buffer and vortex at high speed to resuspended pellet and incubated for 10 minutes at 37°C. The resuspended pellet added with 100 ul of lysis1 and lysis 2 following by mixed and incubated 5 minutes at 65°C. Extraction buffer 100ul then added following by 10 seconds of low speed vortex before centrifuged at the 18,000 g for 3 minutes. Adamax 2 beads 100 μl then added followed by inversion of the tube 10 times and another centrifuged step at the 18,000 g for 3 minutes. An equal volume of
Isopropanol added and centrifuged at 18,000 g for 2 minutes. The supernatant then decanted before DNA washing by 750 ul of 70% ethanol before centrifugation at 18,000 g for 2 minutes. The DNA samples were then air dried upside down for ~30 minutes at RT before being re-suspended in 100 ul of dH2O or 10 mM Tris-HCl. The concentration of total DNA was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and/or Qubit 2.0 Fluorometer (Invitrogen).

**Table 2.3 Saliva buffer mixtures**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 8.0</td>
<td>50 ml</td>
<td>50 mM</td>
</tr>
<tr>
<td>0.5 M EDTA pH 8.0</td>
<td>100 ml</td>
<td>50 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.2 gr</td>
<td>50 mM</td>
</tr>
<tr>
<td>3 M NaCl</td>
<td>33.33 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 ml</td>
<td>1%</td>
</tr>
<tr>
<td>dH2O</td>
<td>top up to 1 L</td>
<td></td>
</tr>
</tbody>
</table>

**2.5.4 Semi-Quantitative Polymerase Chain Reaction (PCR)**

cDNA or DNA originated as previously described were amplified by PCR with primers created using Primer3 ([https://primer3plus.com/primer3web/primer3web_input.htm](https://primer3plus.com/primer3web/primer3web_input.htm)) (Table 2.4). Stock primers at 100 μM were diluted to 5 μM in RNase-free water and stored at -20 °C. The PCR reaction was made up of 12.5 μl of HotStarTaq Master Mix (1000 U), 1 μL forward primer, 1 μL reverse primer, 2 μL cDNA and 8.5 μL RNase-free water, to a total volume of 25 μl. The final concentration in each reaction volume was 1.25 U HotStar Taq DNA polymerase, 1X PCR buffer containing 0.75 mM MgCl2, 100 μM of each dNTP, 0.25 μM of each primer and ~10 ng of cDNA equivalent. The PCR was activated at 94 °C for 10 minutes, denatured at 94 °C for 60 s, annealed at 60-75 °C for 1 minute, extended at 72 °C for 90 s, for a total of 35 cycles, then extended for a final time at 75 °C for 10 minutes before being cooled to 4 °C on a DNA Engine Tetrad 2® Peltier Thermal Cycler (Bio-Rad).
temperature of 60°C was chosen after a temperature gradient was run for the primer pair. After the PCR, 5 μL of 6X Orange Loading Dye (R0631 Thermo Fisher Scientific) was added to each reaction tube and 15 μL loaded on 0.8 to 1% agarose gel containing 0.01% ethidium bromide. The gels were cast on horizontal electrophoresis and ran at 100V for 30-40 minutes. Images were captured using Thermo Scientific MYECL Imager.

**Table 2.4** List of primers used for PCR/qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>target bp</th>
</tr>
</thead>
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<tr>
<td>IL-6</td>
<td>5'-CACTGGCAGAAAACAACCTG-3'</td>
<td>5'-TGACTCATTGACACAGCTCT-3'</td>
<td>191</td>
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<tr>
<td>CXCL10 (IP-10)</td>
<td>5'-AGTGCCATTGACACAGCTCT-3'</td>
<td>5'-TGATGCCATTGACAGCTCT-3'</td>
<td>199</td>
</tr>
<tr>
<td>IL-1B</td>
<td>5'-ACTGAAAGCTCCACCCCTGC-3'</td>
<td>5'-TCCTCCACCTGAGGCTCCC-3'</td>
<td>177</td>
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<tr>
<td>IL-8</td>
<td>5'-CAATTTGCAAGGTATGCT-3'</td>
<td>5'-CCAGTTTTCAGGGCTCCA-3'</td>
<td>185</td>
</tr>
<tr>
<td>PPIA</td>
<td>5'-GTTTCTCGGACATGTCCG-3'</td>
<td>5'-CAATATGCGTGGTCAGTCA-3'</td>
<td>357</td>
</tr>
<tr>
<td>16S RNA 1492 and 27 f</td>
<td>5'-CGGTTACCTGTAGCTGACT-3'</td>
<td>5'-AGAGTATGATMTGGCCTGACG-3'</td>
<td>1500</td>
</tr>
</tbody>
</table>

### 2.5.5 Quantitative Polymerase Chain Reaction (qPCR)

Real-time quantitative PCR (qPCR) was performed using QuantiFast SBYR®Green PCR kit (Qiagen), in duplicate on a Mastercycler® ep realplex (Effendorf) with primers created using Primers from Table 2.4. The qPCR reaction was made up of 12.5 μl 2x QuantiFast SYBR Green PCR Master Mix, 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl cDNA and 9.5 μl RNase-free water, to a total volume of 25 μl. The final concentration in the reaction volume was 1x QuantiFast SYBR Green PCR Master Mix, 0.1 μM of each primer and ~10 ng of cDNA equivalent per reaction. The PCR mix was activated at 95 °C for 15 minutes, denatured at 95 °C for 15 s and annealed/extended at 60 °C for 60 s, for a total of 40 cycles, then a melting curve performed. The annealing temperature of 60 °C was chosen after a temperature gradient was run for each primer pair.
Normalized mean gene expression values were determined from duplicate cycle threshold (Ct) values for each gene and the housekeeping gene peptidylprolyl isomerase A (PPIA). Relative transcript levels were determined by the $2^{-\Delta\Delta Ct}$ method.\(^{(153)}\)

2.6 Transepithelial electrical resistance (TEER) measurement

MOE1a cells $5 \times 10^4$ to $10^5$ in 250 µl were placed in the upper chamber of each well of 24-well Transwell plate with 0.4 um pore polyester membranes (Sigma, Cat no. CLS3413-48EA) and culture medium was changed every three days.

The TEER was then measured every day or based on specific time if the cells stimulated, using the EVOM\(^2\) (World precision instrument) and STX2 electrode at three different sides per Transwell insert. The EVOM\(^2\) produces an AC current that avoids electrode metal deposits and adverse effects on tissue, which can otherwise be caused by a DC current. In addition, resistance readings are unaffected by membrane capacitance and membrane voltage. The STX2 electrode incorporates a fixed pair of probes, 4 mm wide and 1 mm thick. Each probe has an outer and an inner electrode. The outside electrodes are small silver (Ag) pads that pass current through the membrane sample. They are referred to as current electrodes. The inner electrodes are small Ag/AgCl pellet voltage sensors. They are referred to as voltage electrodes (Figure 2.2).

The probe then put in the upper chamber medium for a few minutes and the resistance measurement read at the EVOM\(^2\) display. The value of the chamber treated then subtracted with the blank resistance to obtain the true resistance value of the cultured cell monolayers.
2.7 In vitro wound healing assay and time lapse imaging

This assay aimed to observed cell migration and the rate of gap healing in an artificially ‘wounded’ cell monolayer. Briefly, MOE1a cells were plated in a 12-well tissue culture treated microplates (Falcon) under normal culture conditions (see section 2.2.2) until they reached 100% confluence. Cell medium then removed, and the monolayers were scratched using a 200 µl tip pipette in one straight line to produce a uniform cell-free area. The wells then washed twice to remove any floating cells and debris before stimulating with either HkEc, VSL#3, or both. We generally using 200 moi to stimulate MOE1a with VSL#3 and 20 moi for HkEc. The 200 moi define as number of cells which would stimulated multiply by 200 in 1 cell. Both VSL#3 and HkEc bacterial stocks were prepared on 10^{10} aliquots. The HkEc kindly prepared by Andre Ribeiro Ribeiro and the VSL#3 stocks aliquot based on packages information.
Plate were then incubated in a Live Imaging Zeiss Axiovert microscope with an incubator box set at 37°C and connected to a camera. The camera was set to capture three images per well every 10 minutes for 48 hours. The images were combined to generate movie files and still images extracted at baseline, 6, 12, 24, and 48 hours. The percentage of wound closure was calculated using ImageJ public domain software (https://imagej.nih.gov). The ImageJ-macros is the plug in addition that automatically measured a series of ImageJ commands, including to measure cell-free area on the in vitro wound healing assay. In this studies, we did not use any ImageJ plug in tools since some of the MOE1a cell migrations were more individually, rather than collective which made the automatic measurement difficult.\(^{(154)}\)

2.8 Peripheral blood collection

2.8.1 Ethics

This human study was approved by the Joint University College London (UCL)/University College London Hospitals (UCLH) Committee for the Ethics of Human Research and the NHS National Research Ethics Service, London-Surrey Borders Committee (project number 10/H0806/115).

2.8.2 Saliva collection

Saliva was collected at the same time as the blood samples. About 2-5 ml of unstimulated whole saliva was taken from the participant into the 50 ml universal tubes containing saliva buffer (Table 2.3). The samples then put in the -80°C freezer before samples processing as per protocol of 2.5.3
Figure 2.3 Workflow of *in vitro* scratched 'wound' healing assay using MOE1a cells and time-lapse video microscopy imaging.

Figure 2.4 representative image j on how the in vitro 'wound' closure rate was measure.
2.8.3 Serum collection

Peripheral blood samples of participants (10-50 ml) were collected in 10 ml K2E (EDTA) BD Vacutainer collection tubes (Cat No. 367525-BD). The blood was then transferred into a 15 or 50 ml Falcon tube and centrifuged at 700 g for 20 minutes at RT. The yellowish supernatant then put in 5 ml cryovial tubes and stored at -80º C until needed.

2.8.4 Neutrophil isolation

Neutrophil were isolated from ~7 ml peripheral venous blood samples collected in 10 ml K2E (EDTA) BD Vacutainer collection tubes. The blood was transferred into a 15 ml Falcon tube and 1:10 dilution of 10 % dextran solution (MW 200,000-300,000 (MP Biomedicals) in 0.9% NaCl) added for a final concentration of 1 %. The mixture was left to sediment for 30 minutes at RT. The upper layer containing the leukocytes was removed and layered on top of 5 ml Lymphoprep (1.077 g/ml, Alere LTD, Cat No. 1114547) in a standard 15 ml polypropylene centrifuge tube (SLS, Cat No. BC031) before centrifugation at 400 g for 30 minutes. The enriched neutrophil pellet is collected and the contaminating red blood cells removed using hypotonic lysis. The cell pellet is resuspended in sterile distilled water for 10 seconds followed by the addition of an equal volume of 2x saline solution. The neutrophils are then pelleted at 200 g for 10 mins and washed 2 times in PBS. The neutrophil concentration and viability was measured using an automated cell counter (Biorad TC20) and trypan blue exclusion.
2.9 Clinical study

2.9.1 OLP screening

Methods for this clinical study will specifically written on Chapter 3 along with inclusion and exclusion criteria.

2.9.2 CABRIO study

Methods for this clinical study will specifically written on Chapter 6 along with inclusion and exclusion criteria.

2.10 Data analysis

All data are presented as mean ± SEM using GraphPad Prism 8.0 (GraphPad Software, Inc). Statistical significance was calculated using paired or unpaired two-tailed Student's t-test when 2 groups were analysed. When more than two groups were analysed, statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test or Dunnet's multiple comparison test when a control/reference group was used. Simple linear regression analysis, area under the curve and analysis of covariance (ANCOVA) was used to compare to linear regressions were calculated using GraphPad Prism 8.0.

All clinical data analyse using STATA 15 program (Stata Inc.) with statistical significance was calculated using linear regression and paired or unpaired two tailed Student t-test as instructed by data performance. Specifically, for clinical CABRIO trial data, statistical analysis was performed under statistician supervision according to previous statistical analysis plan (SAP).
CHAPTER 3
OLP PRO-INFLAMMATORY CYTOKINES PROFILE
CHARACTERIZATION

3.1 Introduction

Cytokines and chemokines are small proteins or glycoproteins with molecular weights typically less than 30 kDa that are released by many different cell populations. The main function of these proteins is to trigger communication between different cells and tissue. They are critical in the generation and activation of an effective immune response toward any antigen or pathogen (2). A complex networks of cytokines interact in a dynamic way to regulate the immune response and other biological pathways. Aberrations in the immune response resulting from alterations in cytokine levels have been strongly associated with a host of chronic diseases such as oral lichen planus (OLP). (155)

Previous studies have demonstrated increased NF-kB-dependent cytokines like TNF-α, CXCL10, CXCL8, IL-1B, IL-6 and IFN-γ both systemically and locally in patients with OLP compared to healthy control (HC) subjects. (78, 156-159) Domingues reported detectable TNF-α and high CXCL9 and CXCL10 serum levels were observed in patients with Lichen planus (LP) and suggesting their potential use as markers of the inflammatory status in LP. (31) The similar results published on increased CXCR3 and CXCL10 serum level of OLP patients compared to control group. This research group reported less expressed CXCR3 protein in the membrane and cytoplasm of lymphocytes in the lamina propria of OLP patients. (158) However, CXCL10 was strongly expressed in the membrane and cytoplasm of the lamina propria lymphocytes and the cell membrane of the epithelial cells in OLP patients while in the normal oral mucosa, the CXCL10
staining was weakly positive. The immunoreactive scores showed that the expression level of CXCL10 in the tissue lesions of patients with OLP was significantly higher than that of the normal control group.\(^{(158)}\)

Locally, IFN-γ and IL-33 reported more expressed in OLP lesions than in nonspecific inflammatory lesion samples. Additionally, IFN-γ protein expression was expressed higher in the saliva of OLP group compared to controls.\(^{(37)}\) Similarly, the content of IL-6 and TNF-α in saliva of OLP patients were significantly higher than that in the HC group. However, compared with the erosive type of OLP group, the expression levels of these 2 inflammatory cytokines were similar in the non-erosive OLP.\(^{(30)}\) Additionally, this group reported NF-κB p65 which was located in some intrinsic layers, the epithelial basal layer and spinous layer, and was mainly expressed in the cytoplasm and nucleus of cells.

Furthermore, the reactive oxygen species (ROS) production may also play a role in the pathogenesis of this disease.\(^{(160, 161)}\) Some of the published studies reported different results about various cytokines level differences between OLP versus healthy control (HC) individual.\(^{(162, 163)}\)

In addition, there were some authors reported different association between disease severity and cytokines level either systemic or locally. Differences in reported cytokines level in association with disease severity probably related to different clinical phenotype or cofactor contribution such as systemic diseases or any psychological disorders which underlying OLP etiopathogenesis.\(^{(164)}\) A systematic review by Humberto et al on cytokines as salivary biomarkers in OLP revealed inconsistency between the level of evidence reported by some authors and suggested low evidence and weak grade of recommendation. This might be contributed by the type of the studies in the
systematic review were mainly case control or non-randomised studies.\textsuperscript{(164)} Additionally, the difference might came from different standardized outcome measures.\textsuperscript{(165)} To overcome this issue, some experts generate validated outcome measure for oral mucosal chronic disease including oral lichen planus. The scoring system involved pain, disease severity in matrix column and quality of life scoring. Oral cavity divided in 17 anatomical area which then measured based on its clinical lichen planus manifestation. Based on this scoring system we then aimed this study to validate the level of NFkB-dependent cytokines both systematically and locally on OLP disease severity.

3.1.1 The role of CXCL10 in OLP

The next important thing was role of CXCL10 in oral lichen planus. CXCL10 is a chemokine which is also NF-kB-dependent with main function to attract macrophages to the site of infection. CXCL10 which is also called interferon induced protein 10 (IP-10) led to accumulation of activated lymphocytes via binding to its specific receptor CXCR3.\textsuperscript{(78)} These binding once bound these then initiate the generation and migration of effector T cells following induction of directed migration of lymphocytes to specific sites of lesion or infection. Consequently, the lymphocyte migration causing the infiltration of lymphocytes, and mainly producing local immune responses leading to tissue damage such as seen in diseases like rheumatoid arthritis, multiple sclerosis, and other autoimmune diseases. Those diseases are related with accumulation of CXCR3-expressing Th1 cells in the inflammatory site.\textsuperscript{(158, 166-168)} These inflammatory chemokines are recently associated with OLP disease.\textsuperscript{(25, 44, 158, 169, 170)} Di Lernia (2016) even suggested to target CXCL10 or IFN-γ as the inducing factor for CXCL10 secretion for alternative treatment of lichen planus.\textsuperscript{(171)}
3.1.2 Study aims

This study aimed to:

1. Characterize circulatory and local NF-kB-dependent cytokines profile differences between OLP and HC individual
2. To assess correlation between systemic and locally OLP cytokines level and disease severity based on clinical scoring systems of Guy’s hospital
3. Observing CXCL10 and IFN-γ systemic and local secretion role on the disease severity
4. To learn more about potential cytokines for target treatment of OLP
5. As a base knowledge for our future clinical trial

3.2 Methods

3.2.1 Ethical approval

Full ethical committee approval was obtained under the abnormal genes and proteins in inflammatory bowel disease amendment of Research ethic committee (REC) reference no 10/H0806/115 with IRAs project ID of 28159.

3.2.2 Participant recruitment

3.2.2.1 Clinical diagnostic criteria

The clinical diagnostic criteria is based on WHO histology criteria which included features of a well-defined, band-like zone of inflammatory infiltrate confined to the superficial lamina propria, and liquefaction degeneration of the basal cell layer and clinical sign of OLP as noted and approved by oral medicine consultant in Eastman Dental Hospital. General definitions of the phenotype covered in this study were refer to Eisen criteria (Figure 3.1)
1. A wickham's striae phenotype with interlacing of keratotic, pinhead sized, white, slightly elevated papules or plaque-like discrete of arranged in reticular web-like feature.

2. Erythematous phenotypes refer to superficial reddening of the oral mucosal without any loss of mucosal layer. It could be surrounded by fine white striae.

3. Erosive phenotype presents as either atrophic red areas within the white lesions or well-defined ulceration. Bullous feature included in this phenotype because once it will leave clinical characteristic of ulceration/erosive feature whenever the bulla broke.

Figure 3.1 Eisen’s criteria of oral lichen planus phenotype, A. Very faint reticular lesion, B. Massive reticular web-like lesion (Wickham’s striae) (Image adapted from Greenberg et al 2008) This lesion has started from a more faint structure. C. Plaque-like which also included in reticular phenotype. D, E, F. Faint erythematous lesions, G. Chronic ulcerative phenotype, H and I. Ulcerative lesion surrounded with Wickham’s striae. Those lesions classified as ulcerative phenotype.
3.2.2.2 Participant selection

Participant’s candidate approached by convenience sampling method which aimed to recruit any potential participant available after inclusion and exclusion criteria. A written informed consent form was signed by each participant before they underwent a thorough clinical examination, and blood and saliva sampling. Individual data such as age, gender, smoking history, alcohol consumption, and medical history, and medication was also collected for all volunteers. HC participants were recruited from volunteers who attended Eastman Dental Hospital.

Inclusion criteria:

1. Biopsy-proven diagnosis of OLP
2. Age more than 18 years old
3. Willing to participate into the study

Exclusion criteria:

1. Pregnancy or receiving in-vitro fertilisation (IVF) treatment
2. Less than 18 years of age.

Both OLP and HC participant recruitment was performed in two batches. Batch I was collected over a 6 month period (June to November 2016). Batch II were recruited over 10 months (September 2017 to July 2018). In all cases, participants with OLP lesion were selected from a population attending the Oral Medicine clinic of Eastman Dental Hospital UCLH NHS Foundation Trust according to general inclusions and exclusions criteria above.

3.2.3. Sample collection

All samples collected after the volunteer signed informed consent of the study. Oral examination was completed by main researcher when the volunteer
attend their routine visit. Venous blood then taken by phlebotomy nurses while whole saliva collected. The volunteer asked to spit in to a 50 ml tube containing 2 ml saliva buffer (refer to Chapter 2 section 2.8.3) for 5 minutes or until saliva volume reached 3-5 ml. Blood and saliva subsequently processed according to protocol of serum and saliva extraction listed on Chapter 2 section 2.5.1

3.2.4 Parameters measured

3.2.4.1 Pain numeric rating scale (pNRS)

Pain numeric rating scale (pNRS) is a self-reported pain by individual with OLP where they marked a numerical scale from 0 to 10 with 0 (Figure 3.2) score representing no pain and 10 for the worst imaginable pain they might experience.\(^{172}\)

![PAIN SCORE 0-10 Numerical Rating Scale (NRS)](Image adapted from Marazzu et al 2015)

**Figure 3.2** Pain numeric rating scale (pNRS) (Image adapted from Marazzu et al 2015)

3.2.4.2 Objective measurement

Objective measurement is a cumulative score of inflammation activity and inflammation site score which was collected during oral examination by the clinician. The site measured based on the extent of the lesion found on 17 sites of oral cavity (figure 3.3) and the severity score. 1. keratotic only 2. Erythematous and
3. Erosive/ulcerative phenotype. Whenever the activity appeared together, the lesion would be categorised according to the most severe.

### 3.2.4.3 Oral disease severity score (ODSS)

Oral Disease Severity Score (ODSS) is a scoring system modified from Escudier’s which is recorded on clinical scoring systems for oral mucosal diseases from Guy’s hospital (www.guysandstthomas.nhs.uk). This scoring system divide the oral cavity into 17 sites as showed in figure 3.1. The inflammation activity and site then scored according to presence of lesion at the site with score 0 if there was no lesion, 1 if the lesion affected less than 50% (or unilateral), and 2 if the area affected more than 50% (or bilaterally). For the inflammation activity, score 0 represent no lesion at the site, 1 if the lesion phenotype was mild erythema (e.g. on gingivae, the erythema only happened on the papillae or less than 3 mm along margins), 2 for marked erythema (e.g. full thickness on gingivae, extensive with atrophy or oedema on non-keratinised mucosa), and 3 whenever there was an ulceration (loss of epithelial layer through the surface of lamina propria or deeper tissues).

In general ODSS is accumulation of activity and site score (objective measurement) and pain score.

### 3.2.4.4 Serum and saliva cytokines concentration

Cytokine and chemokines were measured by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method as describe in Chapter 2 section 2.4. Batch 1 (n=27 VS 24 HC) used Peprotech Cat No. 900 K39 Elisa kit to quantify CXCL-10 protein. The second batch (n=45 vs 20 HC) used a combination of multiplex and single cytokine assays (Meso Scale diagnostics pro-inflammatory...
cytokine assay and specific ELISA kit from R&D Systems). The pro-inflammatory cytokines of interest were CXCL8, CXCL10, IL-6, IFN-γ, IL-1B and TNF-α.

3.2.4.5 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) is an important component of the antimicrobial repertoire of neutrophils and macrophages and can be measured by quantifying the amount of hydrogen peroxide (H₂O₂) generated using the reporter dye Amplex® Red (Thermofisher, Cat No. A22188). Neutrophils were isolated from fresh peripheral blood obtained from volunteers. The neutrophil extracted less than 6 hours after the blood sample collected and following the protocol mentioned in Chapter 2 section 2.8.4

3.2.5 Statistic analysis

Data analysed in four different groups as listed in table 3.1. The first group (All samples group) consist all samples from Batch I and Batch II participants regardless systemic diseases or medication background which might affect cytokine serum level. The second group (after exclusion group) involved all participants after exclusion of diseases or drug associated inflammation. Batch I group contain samples from participant in 6 months range of 2016 after exclusion of participant with diseases/medication-associated inflammation. Meanwhile, batch II group occupied by volunteer recruited in 10 months’ time in 2017-2018 after exclusion.

Group division in this analysis was refer to the time of sample collections, in 2016 and 2018 and combination of both for the third group. All subject approached based on the participants selection section. The after exclusion group meant to observed whether the systemic background contributed greatly on the
cytokines level measured. This group also consist of 2016 and 2018 sample collection with exclusion and inclusion criteria based on participant selection section above.

Table 3.1 Groups classification for OLP screening study analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample classified based on</th>
<th>Times range at collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples group</td>
<td>From all recruited participants</td>
<td>all</td>
</tr>
<tr>
<td>After exclusion group</td>
<td>After excluding associated-sistemic diseases</td>
<td>all</td>
</tr>
<tr>
<td>Batch I</td>
<td>Processing with Peprotech kit for CXCL10</td>
<td>6 months range in 2016</td>
</tr>
<tr>
<td>Batch II</td>
<td>Processing with R&amp;D kit</td>
<td>10 months 2017 - 2018</td>
</tr>
</tbody>
</table>

Data was presented with descriptive methods (mean ± SD or median; interquartile range (IQR)) as appropriate calculated using Stata SE 15.1 (StataCorp, US). Statistical significance was estimated by unpaired two-tailed Student’s t-test or Mann Whitney U analysis test depend on data distribution. The Spearman rank correlation test was used to explore associations between levels of circulating serum and saliva cytokines and clinical parameters. Significance level was set at a p value < 0.05.
Figure 3.3 Oral Disease Severity Score (ODSS) sheet. This scoring system divided the oral cavity into 17 sites that were independently scored for site score and activity score. Pain score was also recorded and combined to calculate a total disease activity score. ([www.guysandstthomas.nhs.uk](http://www.guysandstthomas.nhs.uk))

<table>
<thead>
<tr>
<th>Site</th>
<th>Site Score</th>
<th>Activity Score</th>
<th>Pain Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer lips (1)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Inner lips (1)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>R Buccal mucosa (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L Buccal mucosa (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Gingivae (1 each segment)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lower R</td>
<td></td>
<td></td>
<td>0</td>
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<tr>
<td>Lower central</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lower L</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Upper R</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Upper central</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Upper L</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Dorsum of tongue (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>R Ventral tongue (1)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L Ventral tongue (1)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Floor of mouth (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Hard palate (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Soft palate (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Oropharynx (1 or 2)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0</td>
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</tbody>
</table>

Total Score = Site Score + Activity Score + Pain Score

**TOTAL SCORE:**

<table>
<thead>
<tr>
<th>Site Score</th>
<th>Activity Score</th>
<th>Pain Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>5</td>
<td>0</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>7</td>
<td>0</td>
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<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

**Site Score**
- 0 if no lesion
  - For the buccal mucosa
    - 1 if less than 50% of area affected
    - 2 if greater than 50% of area affected
  - For the dorsum of tongue, FOM, hard or soft palate or oropharynx
    - 1 unilateral
    - 2 bilateral

**Activity Score**
- 0 normal/white patch
- 1 mild erythema
- 2 marked erythema
- 3 ulceration at this site

**Pain Score**
Analogues scale from 0 (no discomfort) to 10 (unbearable/the most severe pain imaginable) that they encountered with this condition as an average of the preceding week.
3.3 Results

3.3.1 Data distribution

Data distribution is an important factor in determining mathematical model for data analysis. Figure 3.4 showed normality distribution, which is one way to validate the observed data distribution. For data with normal distribution, $t$ test unpaired two-tail analysis used. While data non-normally distributed, either skewed to the right or left, should be transformed prior to analysis. Log transformation is the most common technique used to transform the data. However, due to most HC data observations being zero (below the range of the assay) it was impossible to use the log transformation method because it violated the mathematical rule of transformation. Therefore, I performed the analysis with a median or non-parametric method if the groups compared are small in size.

For correlation analysis, Pearson's correlation was used when the data normally distributed, otherwise Spearman rank correlation was performed.
Figure 3.4. Data plot normality distribution of OLP and HC for all and after exclusion groups. A, B). OLP and HC data for all samples saliva along with ROS data, respectively.
Figure 3.4 (Continue). Data plot normality distribution of OLP and HC for all and after exclusion groups. C, D). OLP and HC data after exclusion group, a, b, c, d, e, f, g, h for each graph represent data plot normality of serum concentration of TNF-α, CXCL10, CXCL8, IL-6, and CXCL10 saliva along with ROS data,
Figure 3.5. Data plot normality distribution of OLP and HC for Batch I and Batch I groups A, B). OLP and HC data group for batch I.
Figure 3.5. (Continue) Data plot normality distribution of OLP and HC for Batch I and Batch II, C, D). OLP and HC data for batch II. a, b, c, d, e, f, g, h for each graph represent data plot normality of serum concentration of TNF-α, CXCL10, CXCL8, IL-6, and CXCL10 saliva along with ROS data, respectively.
3.3.2 Characteristic demographic of OLP screening study

Characteristic of OLP and HC participants included in this study are detailed in table 3.2. In total this study included 80 OLP histologically proven based on WHO criteria. The criteria included features of a well-defined, band-like zone of inflammatory infiltrate confined to the superficial lamina propria, and liquefaction degeneration of the basal cell layer.\(^{(162)}\) All volunteers fulfilled our exclusion and inclusion criteria. We have 8 (eight) OLP participants with mild dysplasia on biopsy result (2), Diabetes Mellitus (DM2) 2, Crohn’s disease 2, Sarcoidosis 1, and Inflammatory Bowel Disease (IBD) 1. To observe whether those underlying systemic condition role on the systemic or local cytokines level, we then generated one group which exclude the 8 participants above. Four of the excluded participant origins from the Batch I and the other 4 belonging from Batch II.

In summary the typical age presentation of OLP participants was 36 to 86 (mean 60.3, SD 12.1) years old with more frequent participants were female (\(n = 62, 77.5\%\)). Erosive OLP was the most common phenotype recruited (\(n=45, 56.2\%\)) following by erythematous (22, 27.5%) and reticular (13, 16.2%). All OLP participants had multiple-site involvement with most frequent site was on buccal mucosa both left and right within 61 subjects (76.25%) while oropharynx was the lowest with 3 sites (3.85%). Only 6 (7.5%) OLP participants smoke but almost all drink alcohol at least one unit per week. Fifty-nine (73.75%) volunteers using either topical or systemic corticosteroid.

There were 44 HC participants neither with oral mucosal lesions nor acute or chronic systemic diseases. The HC age ranged from 26 to 68 years (mean 38.79, SD 10.29) old with 28 (63.64%) was of female.
Table 3.2 Characteristic demographic of the study

<table>
<thead>
<tr>
<th>Variable</th>
<th>All samples</th>
<th>After exclusion</th>
<th>Batch I (n=27)</th>
<th>Batch II (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OLP (n=80)</td>
<td>HC (n=44)</td>
<td>OLP (n=72)</td>
<td>HC (n=44)</td>
</tr>
<tr>
<td>Age (year ± SD)</td>
<td>60.3 ± 12.1</td>
<td>38.79 ± 10.29</td>
<td>60.65 ± 12.06</td>
<td>39.1 ± 10.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>****</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>OLP (n=27)</td>
<td>HC (n=24)</td>
<td>OLP (n=45)</td>
<td>HC (n=20)</td>
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<tr>
<td></td>
<td>64.15 ± 12.35</td>
<td>35.54 ± 8.22</td>
<td>58.55 ± 11.52</td>
<td>42.7 ± 11.34</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>62 (77.5)</td>
<td>28 (63.64)</td>
<td>55 (76.39)</td>
<td>28 (63.64)</td>
</tr>
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<td>ns</td>
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</tr>
<tr>
<td>Male</td>
<td>18 (22.5)</td>
<td>16 (36.36)</td>
<td>17 (23.61)</td>
<td>16 (36.36)</td>
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<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Smoke (Yes)(%)</td>
<td>6 (7.5)</td>
<td>4 (5.56)</td>
<td>1 (3.70)</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Alcohol (Unit/week)(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 10</td>
<td>60 (82.5)</td>
<td>61 (84.72)</td>
<td>22 (81.48)</td>
<td>60 (82.5)</td>
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<tr>
<td>11 - 20</td>
<td>10 (12.5)</td>
<td>8 (11.11)</td>
<td>4 (14.81)</td>
<td>10 (12.5)</td>
</tr>
<tr>
<td>20 - 30</td>
<td>4 (5.0)</td>
<td>3 (4.17)</td>
<td>1 (3.70)</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>Treatment (Yes)(%)</td>
<td>59 (73.75)</td>
<td>52 (72.22)</td>
<td>17 (62.96)</td>
<td>59 (73.75)</td>
</tr>
</tbody>
</table>

* t-test non parametric, ^ Fisher's exact test, cChi-square test
ns= P > 0.05, * = P ≤ 0.05, ** = P ≤ .
3.3.3 Characteristic clinic scores based on ODSS

Table 3.3 showed that erosive lesion \((n = 45, 56.2\%)\) was the most common type of clinical presentation in this study. This is in contrast with some published article where the reticular form was the commonest found. \(^{(165)}\) We found more erosive lesions in this study because the screening study we performed was a part of the clinical trial screening recruitment. On the trial recruitment we approached patients with a history of erosive lichen planus on their last visit. So, our chance to recruit volunteer with erosive lichen planus was ultimately higher. The second reason was we classified the lesion based on the most severe lesion we found. So, if the reticular lesion appeared along with either erythematous or erosive lesion even though in pinpoint size, then the lesion would be considered as erythematous or erosive phenotype. This method reduces the chance of reticular lesion being counted on the cohort.

Figure 3.6 represent individual distribution of phenotype lesion against disease period in year. Clearly, more severe phenotype associated with longer period of time onset.

Erythematous lesion was the next common lesion found \((n = 22, 27.5\%)\), followed by reticular lesion \((n = 13, 16.2\%)\). In term of inflammation of the sites, both right and left buccal mucosa were most affected in all three phenotypes with 10 (76.6%) and 8 (61.5%), 17 (77.3%) and 19 (86.4%), and 34 (75.5%) and 34 (75.5%) for reticular, erythematous, and erosive phenotype respectively (Figure 3.7). Other affected sites listed on Table 3.2.

It was expected that the pain and ODSS score would be higher on erosive phenotype, although there were some outliers on pain score where three volunteers had a higher pain score even only with reticular lesion. This might
because a real pain feeling was already there but not associated with occurrence of the OLP lesion. Candida infection, burning mouth syndrome, or neurologic abnormality affecting oral tissues were some closely-related disease with OLP that might be the source of the pain. Other explanation was it is difficult to define pain for someone because it would depend on how someone perceived and react to the pain.\(^{(173)}\) As oral pain could be described widely as knife wound, tingling, burning sensation, dull pain, sharp, electric-like, and so on. These would definitively be affecting the volunteer when they put down their pain score accordingly. The discrepancy between high pain score with a clinical severity also mention by Arduino in her publication.\(^{(174)}\)

Statistically, figure 3.8 showed relation between pain and objective score (which was an accumulation of activity and site score) in a positive association direction. However, the wider scattered pain data observed toward the fitted line interpret as weak association with Pearson's coefficient \(r^2 = 0.3947\). This relation become stronger when correlation is associated with ODSS with \(r^2 = 0.5509\). Oral disease severity score (ODSS) is a total score, or the objective measurement and the pain score itself. It is mean that pain is part of the ODSS (part of whole) and would directly affecting mathematical result of the association. Therefore, we consider ODSS association with pain as not true representative.

Table 3.3 Clinical presentation based on phenotype

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Freq.</th>
<th>Percent</th>
<th>Cum.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erosive</td>
<td>45</td>
<td>56.25</td>
<td>56.25</td>
</tr>
<tr>
<td>Erythematous</td>
<td>22</td>
<td>27.50</td>
<td>83.75</td>
</tr>
<tr>
<td>Reticular</td>
<td>13</td>
<td>16.25</td>
<td>100.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>80</td>
<td><strong>100.00</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table presents as stata data table
Figure 3.6. Individual distribution of phenotype lesion against disease period in year

Figure 3.7. Number of participants with OLP phenotype. A. Against pain score, B. ODSS, C. Period (in years)
Table 3.4 Characteristic clinic of OLP participants based on phenotype

<table>
<thead>
<tr>
<th>Variables</th>
<th>Reticular (n=13) (%)</th>
<th>Erythematous (n=22) (%)</th>
<th>Erosive (n=45) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pain score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - &lt;3</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>≥ 3 - &lt;6</td>
<td>0</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>≥6 - 10</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td><strong>Severity score (ODSS)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - ≤ 20</td>
<td>13</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>&gt; 20 - ≤ 40</td>
<td>0</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>&gt; 60 - ≤ 80</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 80</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Duration (year)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - ≤ 10</td>
<td>13</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td>&gt; 10 - ≤ 20</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>&gt; 20 - ≤ 30</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 30 - ≤ 40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>*<em>Site lesion</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer lips</td>
<td>1 (7.69)</td>
<td>1 (4.45)</td>
<td>5 (11.1)</td>
</tr>
<tr>
<td>Inner lips</td>
<td>2 (15.38)</td>
<td>3 (13.63)</td>
<td>7 (15.5)</td>
</tr>
<tr>
<td>Right buccal mucosa</td>
<td>10 (76.92)</td>
<td>17 (77.27)</td>
<td>34 (75.5)</td>
</tr>
<tr>
<td>Left buccal mucosa</td>
<td>8 (61.54)</td>
<td>19 (86.36)</td>
<td>34 (75.5)</td>
</tr>
<tr>
<td>Gingiva lower right</td>
<td>6 (46.15)</td>
<td>13 (59.09)</td>
<td>23 (51.1)</td>
</tr>
<tr>
<td>Gingiva lower central</td>
<td>6 (46.15)</td>
<td>8 (36.36)</td>
<td>22 (48.8)</td>
</tr>
<tr>
<td>Gingiva lower left</td>
<td>6 (46.15)</td>
<td>13 (59.09)</td>
<td>25 (55.5)</td>
</tr>
<tr>
<td>Gingiva upper right</td>
<td>5 (38.46)</td>
<td>14 (63.63)</td>
<td>25 (55.5)</td>
</tr>
<tr>
<td>Gingiva upper central</td>
<td>6 (46.15)</td>
<td>11 (50)</td>
<td>24 (53.3)</td>
</tr>
<tr>
<td>Gingiva upper left</td>
<td>4 (30.77)</td>
<td>14 (63.63)</td>
<td>24 (53.3)</td>
</tr>
<tr>
<td>Dorsum tongue</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (15.5)</td>
</tr>
<tr>
<td>Right ventral tongue</td>
<td>4 (30.77)</td>
<td>4 (18.18)</td>
<td>13 (28.8)</td>
</tr>
<tr>
<td>Left ventral tongue</td>
<td>2 (15.38)</td>
<td>1 (4.45)</td>
<td>13 (28.8)</td>
</tr>
<tr>
<td>Floor of the mouth</td>
<td>2 (15.38)</td>
<td>1 (4.45)</td>
<td>3 (6.6)</td>
</tr>
<tr>
<td>Hard palate</td>
<td>1 (7.69)</td>
<td>5 (22.72)</td>
<td>11 (24.4)</td>
</tr>
<tr>
<td>Soft palate</td>
<td>0 (0)</td>
<td>3 (13.63)</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>0 (0)</td>
<td>1 (4.45)</td>
<td>2 (4.4)</td>
</tr>
</tbody>
</table>

*All participants had lesions at more than one site, 5 (6.25%) involve either cutaneous or genital lesions or both
3.3.4 Gender and age association with cytokines level

Table 3.5 Revealed that in term of gender there were no statistical differences between all cytokines studied. However, the CXCL8 from the table showed wide confidence interval (CI) which statistically interpret as the test true value generated from the model was less precise value. The reason for this wide CI might because there were two batch cytokines analysis with different kit used. Those two kits although using the same principle of sandwich Elisa, but have different sensitivity which lead to different outcome value Nevertheless, these data suggested that all cytokines investigated demonstrated that there were no differences between male and female cytokines level.

When serum and saliva cytokines level and H$_2$O$_2$ generation of OLP volunteer were compared in terms of age group, again there were no statistical
differences. Age for comparison divided in four group categories. This classification was based on hypothesis that human biology and physiology basically changing rendering to age. The group categorical then refer to WHO recommendation. Group zero involved volunteer with age ≥ 20 to ≤ 40 (n = 6, 7.5%), group 1 with age ≥ 41 to ≤ 60 (n = 34, 42.5%), group 2 for age≥ 61 to ≤ 80 (n = 36, 45%), and group 3 for age ≥ 81 (n = 4, 5%), years old. The Kruskal-Wallis test principally is a more general form of t test and a mathematical extension of the Mann-Whitney test. The chi-squared from Kruskal-Wallis test revealed that cytokines level of CXCL8, CXCL10 serum and saliva, IL-6, IFN-γ, and TNF-α and volunteer age were not statistically significant (P value and Chi-squared value of interest cytokines was listed on table 3.6)

**Table 3.5 OLP** Serum and saliva cytokines and hydrogen peroxide generation compared between genders.

<table>
<thead>
<tr>
<th>Parameter measured (n = 80,  F =68, M= 12)</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0.4246 (ns)</td>
<td>36.47 - 53.21</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0.2273 (ns)</td>
<td>5.07 - 35.83</td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.4458 (ns)</td>
<td>44.24 - 201.16</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.4939 (ns)</td>
<td>2.82 - 7.12</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.1504 (ns)</td>
<td>0.39 - 4.62</td>
</tr>
<tr>
<td>IL-1B</td>
<td>0.4791 (ns)</td>
<td>6.75 - 21.11</td>
</tr>
<tr>
<td>ROS</td>
<td>0.4672 (ns)</td>
<td>40.46 -41.76</td>
</tr>
<tr>
<td>CXCL10 Saliva</td>
<td>0.03236 (ns)</td>
<td>203.34 - 276.54</td>
</tr>
</tbody>
</table>

CI: Confidence interval, ns= P > 0.05, *= P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ .0001
ns=non-significant
3.3.5 Disease duration and association with cytokines level

It was interesting to see whether the disease period contribute to the high level of NF-kB-independent cytokines in OLP. Serum and saliva cytokines level and \( \text{H}_2\text{O}_2 \) generation were then compared in terms of disease period categories. The disease period for comparison divided in four groups. The group zero involved volunteer with period of \( \geq 0 \) to \( \leq 5.9 \) \((n = 44, 55\%)\), group 1 with period of \( \geq 6 \) to \( \leq 10 \) \((n = 26, 32.5\%)\), group 2 for period of \( \geq 11 \) to \( \leq 20 \) \((n = 7, 8.75\%)\), and group 3 for period of \( \geq 20 \) \((n = 3, 3.75\%)\) range in years. The chi-squared from Kruskal-Wallis test revealed that cytokines level of CXCL8, CXCL10 serum and saliva, IL-6, IFN-\( \gamma \), and TNF-\( \alpha \) of OLP and HC volunteer were not statistically significant \((P\) value and Chi-squared value of interest cytokines was listed on table 3.7)

<table>
<thead>
<tr>
<th>Variable measured</th>
<th>(P) value</th>
<th>Chi-squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 80, F = 68, M = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>0.1094 (ns)</td>
<td>6.79 with 3 df</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0.0789 (ns)</td>
<td>6.05 with 3 df</td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.3838 (ns)</td>
<td>3.05 with 3 df</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.2295 (ns)</td>
<td>4.31 with 3 df</td>
</tr>
<tr>
<td>IFN-( \gamma )</td>
<td>0.1191 (ns)</td>
<td>5.85 with 3 df</td>
</tr>
<tr>
<td>IL-1B</td>
<td>0.7052 (ns)</td>
<td>1.40 with 3 df</td>
</tr>
<tr>
<td>ROS</td>
<td>0.5897 (ns)</td>
<td>1.92 with 3 df</td>
</tr>
<tr>
<td>CXCL10 Saliva</td>
<td>0.0489 (*)</td>
<td>7.86 with 3 df</td>
</tr>
</tbody>
</table>

\(n\) = subject number, \(F\) = Female, \(M\) = Male, \(df\) = degree of freedom, \(ns\) = non significant
3.3.6 OLP and HC serum and saliva cytokines and ROS concentration

Taking together, the levels of NF-kB-dependent serum cytokines and CXCL10 saliva cytokines displayed significant differences compared to cytokines level of HC from all batches although variety p value expressed (Table 3.8). IL-1B different significantly only on Batch II where the data samples showed more consistent scatter around 0.2 pg./ml compared to the other batches (Figure 3.9).

In addition, the respiratory burst which constitutes the neutrophils major defence mechanism against microbes was shown significant differences compare to HC (Figure 3.10)

<table>
<thead>
<tr>
<th>Variable measured</th>
<th>P value</th>
<th>Chi-squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 80, F =68, M= 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.2430(ns)</td>
<td>4.18 with 3 df</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0.5668(ns)</td>
<td>2.83 with 3 df</td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.4125(ns)</td>
<td>2.87 with 3 df</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.8754(ns)</td>
<td>0.69 with 3 df</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.0595(ns)</td>
<td>7.42 with 3 df</td>
</tr>
<tr>
<td>IL-1B</td>
<td>0.1503(ns)</td>
<td>5.31 with 3 df</td>
</tr>
<tr>
<td>ROS</td>
<td>0.221(ns)</td>
<td>4.40 with 3 df</td>
</tr>
<tr>
<td>CXCL10 Saliva</td>
<td>0.1619(*)</td>
<td>5.14 with 3 df</td>
</tr>
</tbody>
</table>

n=subject number, F= Female, M= Male, df= degree of freedom, ns = non significant
3.3.7 Circulating and saliva cytokines, and ROS production in association with clinical phenotype

In general, all cytokines investigated demonstrating elevation in accordance to severity of lesion, apart from CXCL8 which was decreased in erosive lesion compared to erythematous phenotype (Figure 3.11). Furthermore, it is interesting to note that in all phenotypes CXCL10 circulating serum levels became the highest cytokine, suggesting the important role of this protein in OLP etiopathogenesis.

There were no differences between phenotype regarding the cytokine's saliva concentration, However, the CXCL10 local secretion demonstrated lower level compared to erythematous and erosive lichen planus (insert graph on figure 3.11).
Table 3.8 OLP and HC serum and saliva cytokines profile. TNF-α, CXCL10, CXCL8, IL-6, IFN-γ, IL-1β serum cytokines level, CXCL10 saliva cytokine level measured in pg/ml, and ROS produced level measured in H$_2$O$_2$ production.

<table>
<thead>
<tr>
<th>OLP vs HC parameter measured</th>
<th>ttest value (p value significant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All samples (n=80 OLP vs 44 HC)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.0000 (***)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0.0000 (***)</td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.0000 (****)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.0000 (****)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.0330 (*)</td>
</tr>
<tr>
<td>IL-1B</td>
<td>0.8733 (ns)</td>
</tr>
<tr>
<td>HC ROS</td>
<td>0.1218 (ns)</td>
</tr>
<tr>
<td>CXCL10 Saliva</td>
<td>0.000 (****)</td>
</tr>
</tbody>
</table>
Figure 3.9 Cytokines serum level for different batch analysis A). TNF-α, B). CXCL10, C). CXCL8, D). IL-6, and E). IFN-γ showed significant increase in OLP serum secretion level consistently on all batch compare to healthy control (HC) F). IL-1β cytokine secretion of OLP volunteer only different significantly on batch two analysis group compare to healthy control (* p < 0.05).
Figure 3.10 Local CXCL10 secretion level and hydrogen peroxide production (A) CXCL10 saliva secretion significantly different compared to healthy control (*p < 0.01) and ROS generation which only demonstrated significant different from healthy control in batch II analysis (*p < 0.05).
Figure 3.11 Circulatory cytokines secretion level over phenotypes. Graph from batch II analysis. The insert graph was the CXCL10 saliva distribution which demonstrated lower concentration on reticular phenotype.
Figure 3.12 Matrix correlation between serum and saliva cytokines and ROS production on clinical variables. A). Association between pain and cytokines level B). Association between objective score and cytokines level, C). Association between ODSS and cytokines score. Data analyse using Spearman correlation.
3.3.8 Circulating cytokines serum and saliva, ROS production association with clinical variables of pain, objective scores, and severity (ODSS) score.

CXCL10 serum cytokines showed association toward a positive direction between pain score as demonstrated both in batch I and II analysis even though there was no significant statistically (Table 3.9). However, with the objective measurement and ODSS scores there were positive relation reached statistical significance in batch one and batch II ($P = 0.0473$, and $P < 0.0230$, respectively). Interestingly, CXCL10 saliva cytokines also demonstrated positive association for all variables measured even though none of this associations significant statistically.

Interferon gamma which is a strong inducer for CXCL10 protein showed positive correlation between pain, objective measurement and ODSS for all three-group analysis (all samples, samples with exclusion, and batch II analysis- we did not do any IFN-γ analysis for batch one samples). Specifically, for pain, the mathematical model for the three groups were significant statistically.

Figure 3.13 demonstrated individual summary relation of pain, objective measurement, and ODSS against NF-kB-dependent cytokines investigated. Both CXCL10 revealed consistently strong association on pain, objective measurement and ODSS with rho value of 0.3, 0.38 and 0.44, respectively.

Additionally, when correlating circulating CXCL10 serum and CXCL10 saliva the association was strong and toward the positive direction with rho ($r^2$) value 0.4275. Scattered CXCL10 saliva value closer to the fitted line was represent for strong association (Figure 3.13).
Table 3.9 Spearman correlation analysis between OLP serum and saliva cytokines, of all samples, after biopsy and diseases associated exclusion, batch I, II samples analysis and Pain, Objective score and ODSS scores

<table>
<thead>
<tr>
<th>Variable correlated</th>
<th>Spearman correlation (n = 72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
</tr>
<tr>
<td>TNF-α vs Pain</td>
<td>0.0201 (0.87_ns)</td>
</tr>
<tr>
<td>TNF-α vs Objective</td>
<td>-0.2863 (0.0148_* )</td>
</tr>
<tr>
<td>TNF-α vs ODSS</td>
<td>-0.2073 (0.08_ns)</td>
</tr>
<tr>
<td>CXCL10</td>
<td></td>
</tr>
<tr>
<td>CXCL10 vs Pain</td>
<td>-0.0355 (0.77_ns)</td>
</tr>
<tr>
<td>CXCL10 vs Objective</td>
<td>-0.3347 (0.0041_** )</td>
</tr>
<tr>
<td>CXCL10 vs ODSS</td>
<td>-0.2951 (0.0118_* )</td>
</tr>
<tr>
<td>CXCL8</td>
<td></td>
</tr>
<tr>
<td>CXCL8 vs Pain</td>
<td>0.1144 (0.34_ns )</td>
</tr>
<tr>
<td>CXCL8 vs Objective</td>
<td>0.1304 (0.27_ns )</td>
</tr>
<tr>
<td>CXCL8 vs ODSS</td>
<td>0.1640 (0.17_ns )</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
</tr>
<tr>
<td>IL-6 vs Pain</td>
<td>-0.1371 (0.25_ns )</td>
</tr>
<tr>
<td>IL-6 vs Objective</td>
<td>-0.3539 (0.0023_** )</td>
</tr>
<tr>
<td>IL-6 vs ODSS</td>
<td>-0.2607 (0.0270_* )</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
</tr>
<tr>
<td>IFN-γ vs Pain</td>
<td>0.3824 (0.0093_** )</td>
</tr>
<tr>
<td>IFN-γ vs Objective</td>
<td>0.1396 (0.36_ns )</td>
</tr>
<tr>
<td>IFN-γ vs ODSS</td>
<td>0.1868 (0.22_ns )</td>
</tr>
<tr>
<td>IL-1B</td>
<td></td>
</tr>
<tr>
<td>IL-1B vs Pain</td>
<td>0.1717 (0.15_ns )</td>
</tr>
<tr>
<td>IL-1B vs Objective</td>
<td>0.1503 (0.20_ns )</td>
</tr>
<tr>
<td>IL-1B vs ODSS</td>
<td>0.1656 (0.16_ns )</td>
</tr>
<tr>
<td>ROS</td>
<td></td>
</tr>
<tr>
<td>ROS vs Pain</td>
<td>0.0446 (0.71_ns )</td>
</tr>
<tr>
<td>ROS vs Objective</td>
<td>0.0982 (0.44_ns )</td>
</tr>
<tr>
<td>ROS vs ODSS</td>
<td>0.0960 (0.45_ns )</td>
</tr>
<tr>
<td>CXCL10 Saliva</td>
<td></td>
</tr>
<tr>
<td>CXCL10 saliva vs Pain</td>
<td>-0.0290 (0.82_ns )</td>
</tr>
<tr>
<td>CXCL10 saliva vs Objective</td>
<td>-0.0277 (0.83_ns )</td>
</tr>
<tr>
<td>CXCL10 saliva vs ODSS</td>
<td>-0.0163 (0.90_ns )</td>
</tr>
</tbody>
</table>
3.4 Discussion

This study aimed to 1). Investigate NF-kB dependent cytokines and ROS differences between OLP and HC volunteers, 2). Observe whether there was any association between the level of the cytokine and the clinical parameter, 3) find out potential target for alternative treatment of OLP.

OLP aetiology is not completely understood. The prevailing theories resolve around dysregulated immune-mediated cytotoxicity activated by exogenous triggers. This can be amplified through the release of pro-inflammatory cytokines such as TNF-α, CXCL10, CXCL8, IL-6, IFN-γ, and IL-1β. These cytokines depend on NF-kB which is a crucial transcription factor responsible for activating pro-inflammatory genes.\(^2\) In
OLP, NF-kB and its associated cytokines has been investigated intensively. \(^{(169, 176-179)}\)

Santoro et al reported a positive association between activated NF-kB in keratinocytes and activation and recruitment of cytotoxic T cells in oral and cutaneous lichen planus.\(^{(178)}\) Another study also reported high level of NF-kB-p65 in the nuclei of both basal and supra basal epithelial keratinocytes and infiltrated lymphocytes in OLP lesion.\(^{(179)}\) Furthermore, an abundant data demonstrate the imbalance of NF-kB-dependent cytokines between OLP patients and HC, either from circulatory or locally from saliva or oral mucosal tissue.\(^{(6, 7, 23, 25, 156-158, 162, 169, 170, 176, 180, 181)}\)

**Figure 3.14** NF-kB role in inducing inflammation. In OLP disease the NF-kB dependent cytokines well known to trigger and amplified severity of the disease. NF-kB activation via diverse stimuli such as cytokines receptors, TNF receptors or pathogen recognition receptors (PPRs) would initiate a range genes involved in inflammatory complexes. It then would increasing cytokines and chemokines adhesion molecules as well as modulating apoptosis, morphogenesis and differentiation. Image adapted from Liu et al (2017)\(^{(2)}\)
Higher level of circulating and salivary NF-kB pro-inflammatory cytokines of OLP volunteer in this study, along with other studies (23, 25, 78, 155-158, 162, 169, 176, 181, 182), add more evidences of NF-kB role in OLP. Overexpression of this transcription factor has been suggested as the cause of chronic inflammation in OLP via secretion of associated cytokines. (2) Activation of this inducible transcription factor family involves two main signalling pathways, canonical (classic) and non-canonical (alternative) pathway. The canonical pathway activate by a range diverse stimulus such as different cytokine receptors, pattern recognition receptors (PPRs), TNF receptor (TNFr) superfamily members, and T-cell receptor (TCR) and B-cell receptor. This pathway mainly responsible for transcriptional induction of pro-inflammatory cytokines, chemokines, and additional inflammatory mediators in various types of innate immune cells. While the non-canonical pathway perform its function as a supplementary signalling axis in conjunction with canonical NF-kB pathway in regulation of specific function of the adaptive immune system. (184) Inactive NF-kB abundantly found in the cytoplasm via interaction with inhibitor molecules of the IkB family. The common mechanism for canonical pathway activation is by proteasomal IkB degradation through its site-specific phosphorylation by multi-subunit IkB kinase (IKK) complex. In turn, IKK can be activated with a range stimulus including cytokines, growth factors, mitogens, stress agents and microbial components. (2, 184-186)

The innate immune cells such as macrophages, dendritic cells, neutrophils, and some non-immune cells such as epithelial and keratinocytes express PRRs which recognise various microbial components via pathogen-associated molecule patterns (PAMPs) (72) Additionally, the PRRs also recognise molecules secreted by necrotic and damaged tissues named damage-associated molecular patterns (DAMPS). One well investigated family of PRRs is toll-like receptors (TLRs). In
human, there were ten different TLRs that have been discovered with almost all their specific ligands. These TLRs commonly divided in two class based on their localization. The TLRs 1, 2, 4, 5, 6, and 10 are the protein located at the cell membrane (extracellular), and others (TLRs 3, 7, 8, and 9) are attached in the endosome (intracellularly).

Recently, it has been suggested that microorganisms contributed to trigger and amplify the severity of OLP disease. The evidence base demonstrated via investigation the presence of microorganism extracellular or intracellularly.\(^{45, 100, 156}\) Furthermore, some authors reported upregulation of TLR2, TLR4, TLR5, and TLR9 at the PBMC, salivary, and mucosal tissue of OLP individual.\(^{31, 45, 48, 85}\)

Upregulated NF-κB would release downstream of NF-κB–dependent cytokines secretion such as demonstrated in our study. From the four different analysis in our study, TNF-α, CXCL10, CXCL8, IL-6, and IFN-γ serum levels and CXCL10 saliva cytokine level revealed statistically significant between OLP and HC. Group I was all volunteer recruited in this study, while group excluded 8 volunteer which have disease-/medication associated-inflammation or with atypia/dysplastic changing on their biopsy results. The analysis of group I all samples and group II with exclusion samples did not show any difference which suggested that those molecules elevated on individual with OLP, hence disease/medication-associated inflammation do not contribute greatly. However, due to the small sample size of excluded samples, and great variability of the exclusion factor in group II, further investigation needs to be done to confirm this conclusion.

Furthermore, IL-1B and ROS production only showed significant value at batch II. Batch I and batch II samples origin from different time point recruitment which in turn analyse with different method of cytokine quantification and the kit used.
batch I, the point of recruitment performed in sixth month as a pilot project for cytokines profiling on OLP individual. Results of this batch needed for in vitro study and as a basic data to plan a clinical trial. In Batch I analysis, cytokine measurement performed with sandwich ELISA method using Peprotech company kit for CXCL10 as our unpublished data refer this kit sensitive for CXCL10 cytokine. While the other cytokines quantified using R&D kit. At the second batch, TNF-α, IL-6, IL-1B and IFN-γ measured using Meso scale diagnostic (MSD) pro-inflammatory assay machine and kit while the CXCL8 and 10 serum dan CXCL10 saliva measured by R&D kit. The same significant differences for all investigated cytokines apart from IL-1B again confirm that the level of those cytokines different from HC regardless the method or kit used to quantify the molecules.

In clinical parameters, CXCL10 serum in batch I and II showed a positive relation for all clinical parameters investigated namely pain, objective measurement, and ODSS (as an accumulation of pain and objective measurement). While on all and with exclusion group, the relation also exists even though its direction going to negative relation. This might be associated with the huge different concentration from the different batch analysis that makes the gap range in both groups analyse higher and makes the statistical analysis skewed. For this analysis, we more confidence with the mathematical measurement of batch I and II where each group shared the same range concentration standard.

There were two interesting point from this association analysis. First, the CXCL10 saliva concentration showed the same association with CXCL10 serum. This is in agreement with other studies which reported some NF-kB-dependent cytokines such as IL-6, TNF-α, and IL-8 serum were correlated in positive manner with saliva cytokines level.\textsuperscript{7, 156, 180} Secondly, IFN-γ which is an inducer factor for CXCL10 (also
named IP-10-interferon gamma- induced protein 10) demonstrated a positive relation for all groups’ analysis, with specifically for pain score which showed significant statistic result.

Our results revealed that for age, gender, and disease range period have no association with the cytokines level. However, almost all cytokines levels investigated were higher in more severe phenotype, with the CXCL10 serum level showed as the highest level of cytokines which suggest the important role of this molecule.
CHAPTER 4.
ISOLATION AND CHARACTERISATION OF VSL#3 SINGLE SPECIES

4.1 Introduction

It has been long known that some microorganisms have symbiotic mutualism relationship with humans. The beneficial effects of regular consumption of fermented dairy products have been appreciated for centuries.\(^1\)\(^3\) The Russian immunologist, Ellie Metchnikoff has risen awareness about the possible positive effect of these microorganisms on human health. His statement that “the dependence of intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” marked the point of probiotic definition (Figure 4.1).\(^187\)

![Figure 4.1. Chronological sequence of probiotic development through the era of next-generation probiotic. Adapted from O-Toole et al.\(^3\)](image)

More than a hundred years since that time, the World Health Organization (WHO) define probiotics as the living microorganisms which when administered in adequate amounts will confer health benefits to the host.\(^188\)\(^188\)\(^188\)\(^188\)\(^188\)\(^187\)\(^186\)\(^185\)\(^184\)\(^184\)\(^183\)\(^182\) The pubmed has indices more than 24,000 research
articles about probiotics. Those publications report a huge range of benefits attributed to probiotics in a host of different conditions.

Recently, probiotic cocktails comprising various strain are used to improve effectiveness over single strains.\(^{189-200}\) The current marketed mixtures consist of up to 31 strains and up to 9 x10\(^{11}\) billion colony forming unit (CFU) living microorganisms per daily dosage (Table 4.1). These mixtures are thought to confer an additional health benefit since different strains establish specific beneficial effects to their host or targeted conditions. As an example \textit{Lactobacillus gasseri}, \textit{Lactobacillus salivarius}, and \textit{Lactobacillus johnsonii} are not capable of generating folate while other \textit{Lactobacillus} species such as \textit{Lactobacillus reuteri}, \textit{Lactobacillus acidophilus}, \textit{Lactobacillus plantarum}, \textit{Lactobacillus bulgaricus}, and \textit{Bifidobacterium longum} synthesise this molecule.\(^{201-205}\). Strain specific differences are also affect the regulation of epithelial barrier function and immune responses. For instance, \textit{Lactobacillus plantarum} PBSO67, \textit{Lactobacillus rhamnosus} PBSO70, and \textit{Bifidobacterium longum} subs. \textit{Longum} PBS108, and \textit{Lactobacillus reuteri} PBS072 can stimulate IL-4 release and reduce TNF-\(\alpha\) levels on human intestinal HT-29 cell line. Meanwhile \textit{Lactobacillus fermentum} PBS073 and \textit{Bifidobacterium animalis} subsp. \textit{Lactis} PBS067 have very little effect on the same cytokines.\(^{206}\) Another benefit in using probiotic mixtures are the symbiotic relationships between species that may exist. Chapman (2011) concluded that probiotic cocktails are more effective compared to the single species. He also reported that poly-biotic mixtures might be more effective against different pathogens and may have wider spectrum of action compare to single preparation, even though the demonstrated effect may be due simply to a greater concentration of probiotics in the cocktails.\(^{207, 208}\)
Because individual probiotic strains perform different metabolic and immunological functions, variability in the amount of a specific strain in the final blend is likely to impact on mixture functionality. However, these beneficial effects have been observed for only a limited number of strains whereas other strain of even the same species cannot be presumed to demonstrate the same activities information as to whether mixing of strains results in synergistic or even additive effects in terms of bioactivity or in reduced efficacy due to mutual inhibition by the component strains.\(^{(208)}\)

One of the most commonly used poly-biotic is VSL#3 which is a lyophilized mixture consisting of eight different Gram-positive organisms *Lactobacillus acidophilus* (BA05), *Lactobacillus delbrueckii* subsp. *Bulgariicus* (BD08) (reclassified as *lactobacillus helveticus*_product label information), *Lactobacillus paracasei* (BP07), *Lactobacillus plantarum* (BP06), *Bifidobacterium longum* (BL03), *Bifidobacterium infantis* (BI04) (BL03 and BI04 reclassified as *B. animalis* subsp. *lactis*_product label information), *Bifidobacterium breve* (BB02), and *Streptococcus thermophilus* (BT01). It is marketed in the UK in capsule and powder form contain up to 450 billion bacteria. Until 2019, VSL#3 is available on British National Formulary (BNF) for maintenance of remission of ileoanal pouchitis only in adults as induced by antibiotics.\(^{(209-211)}\) However, due to weak evidence that the products are clinically effective, VSL#3 along with the Vivomix (contains similar 8 strains and 450 billion CFU microorganism) reported to have been removed from the drug tariff after a review by Advisory committee on borderline substance (ACBS) and should not be routinely prescribed in primary care. \(^{(212)}\)
<table>
<thead>
<tr>
<th>Brand name</th>
<th>Number of strain</th>
<th>Probiotic strains</th>
<th>Daily</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSL#3</td>
<td>8</td>
<td><em>Lactobacillus acidophilus</em>, <em>Lactobacillus paracasei</em>, <em>Lactobacillus plantarum</em>, <em>Bifidobacterium breve</em>, <em>Bifidobacterium infantis</em>, <em>Bifidobacterium longum</em>, <em>Lactobacillus delbrueckii</em> subsp. <em>bulgaricus</em>, <em>Streptococcus thermophilus</em></td>
<td>90</td>
<td>Powder**</td>
</tr>
<tr>
<td>Wood multi strain</td>
<td>11</td>
<td><em>Lactobacillus acidophilus</em>, <em>Bifidobacterium longum</em>, <em>Bifidobacterium infantis</em>, <em>Bifidobacterium bifidum</em>, <em>Bifidobacterium lactis</em>, <em>Lactobacillus Salivarius</em>, <em>Lactobacillus casei</em>, <em>Lactobacillus rhamnosus</em>, <em>Lactobacillus reuteri</em>, <em>Lactobacillus helveticus</em>, <em>Lactobacillus rhamnosus</em></td>
<td>0.1</td>
<td>Capsule</td>
</tr>
<tr>
<td>Elixia</td>
<td>11</td>
<td><em>Lactobacillus acidophilus</em>, <em>Bifidobacterium longum</em>, <em>Bifidobacterium breve</em>, <em>Bifidobacterium bifidum</em>, <em>Bifidobacterium lactis</em>, <em>Lactobacillus Salivarius</em>, <em>Lactobacillus casei</em>, <em>Lactobacillus rhamnosus</em>, <em>Lactobacillus reuteri</em>, <em>Lactobacillus helveticus</em>, <em>Lactobacillus rhamnosus</em></td>
<td>50</td>
<td>Capsule</td>
</tr>
<tr>
<td>Bio-Kult probiotic</td>
<td>14</td>
<td><em>Bifidobacterium longum</em> PXN®30**, <em>Bifidobacterium breve</em> PXN®25**, <em>Bifidobacterium bifidum</em> PXN®23**, <em>Bifidobacterium infantis</em> PXN®22**, <em>Lactobacillus plantarum</em> PXN®47**, <em>Lactobacillus casei</em> PXN®57**, <em>Lactobacillus rhamnosus</em> PXN®54**, <em>Lactobacillus acidophilus</em> PXN®35**, <em>Lactobacillus salivarius</em> PXN®57**, <em>Lactobacillus helveticus</em> PXN®45**, <em>Lactobacillus lactis</em> spp.lactis PXN®63**, <em>Streptococcus thermophilus</em> PXN®66**</td>
<td>0.4</td>
<td>Capsule</td>
</tr>
<tr>
<td>Cultures complex Nutri</td>
<td>15</td>
<td><em>Lactobacillus acidophilus</em>, <em>Bifidobacterium longum</em>, <em>Bifidobacterium lactis</em>, <em>Bifidobacterium breve</em>, <em>Bifidobacterium bifidum</em>, <em>Lactobacillus casei</em>, <em>Lactobacillus rhamnosus</em>, <em>Lactobacillus reuteri</em>, <em>Lactobacillus helveticus</em>, <em>Lactobacillus plantarum</em>, <em>Lactobacillus bulgaricus</em>, <em>Lactobacillus brevis</em></td>
<td>4</td>
<td>Capsule</td>
</tr>
<tr>
<td>Probiotic strains</td>
<td></td>
<td><em>Lactobacillus acidophilus</em>, <em>Bifidobacterium longum</em>, <em>Bifidobacterium lactis</em>, <em>Bifidobacterium breve</em>, <em>Bifidobacterium bifidum</em>, <em>Lactobacillus casei</em>, <em>Lactobacillus rhamnosus</em>, <em>Lactobacillus reuteri</em>, <em>Lactobacillus helveticus</em>, <em>Lactobacillus plantarum</em>, <em>Lactobacillus bulgaricus</em>, <em>Lactobacillus brevis</em></td>
<td>1.725</td>
<td>Capsule</td>
</tr>
</tbody>
</table>
Apart from ileoanal pouchitis, VSL#3 has been studied both clinically and experimentally in other diseases. A meta-analysis conducted by Mardini (2014) using five studies consisting of 144 ulcerative colitis subjects who consumed VSL#3 demonstrated reduced symptoms in 75% of patients compared with only 25% in the placebo cohort. In addition to effects in IBD, Wong et al reported a beneficial effect of VSL#3 in inflammatory bowel syndrome (IBS) with significance improvement in abdominal pain, distention intensity, increased release of salivary morning melatonin, increase in satisfaction with bowel movements and quality of life. Angurana et al also reported the significant decrease in pro-inflammatory cytokines and increase for the anti-inflammatory cytokines in a cohort of children with severe sepsis after 7 days of VSL#3 consumption.

The credibility of poly-biotic VSL#3 as a beneficial supplement is also supported from studies on liver disease, cirrhosis, multiple sclerosis, and chronic pelvic syndrome. It has also been shown to be capable of changing the composition of the microbiome in some conditions.

Interestingly, Chen et al suggested that *Bifidobacterium longum* and VSL#3 demonstrated the similar capability in inhibit HMGB1 (High mobility group box 1) secretion and consequently mediated gut barrier dysfunction. While Hormannsperger suggested the *Lactobacillus casei* has a similar effect as whole VSL#3 in inhibiting CXCL10 release.

Although VSL#3 mixture has been investigated intensively for a wide range of clinical situations, the single species components from this poly-biotic are rarely characterized. In this chapter, I attempted to isolate and study the 8 different strains contained in VSL#3. As far as I am aware, this might be the first characterisation of the single species of VSL#3 derived directly from the marketed product, as other single species probiotic characterisation obtained their bacterial stocks from
commercial clones.\(^{(222, 223)}\) Biagioli 2019 performed direct culture from the marketed sachets by inoculated lyophilized probiotic microorganism on de Man-Rogosa Sharpe (MRS) broth and plating by serial 10-fold dilution onto MRS-agar containing 0.05% L-cysteine-HCl for 48 hours for Colony forming unit counting.\(^{(224)}\)

4. 2. Material and Methods

4.2.1 Microbiological culture

VSL#3 powder bought online from VSL#3 website (Ferring Pharmaceuticals, UK batch no 507132 expired date 07/2017) was resuspended in 50 ml phosphate-puffered-saline (PBS) (Gibco, cat no.20012-068) and centrifuged at 2000 g for 10 minutes. The pellet was re-suspended in 50 ml PBS and washed a further three times. VSL#3 diluted in PBS to a final stock concentration of \(1 \times 10^8\) cfu/ml based on number of cfu written on the label. VSL#3 stock was either heat killed, formaldehyde fixed, or left viable. Heat killed protocol was performed at 90°C for 30 minutes and formaldehyde fixing was performed using 5% formaldehyde (Sigma, 252549.) for 3 hours at 4°C. Fixed bacteria washed three times in PBS and centrifuge at 2000 g 10 minutes. Killing efficiency was verified by plating 10 µl of stock on BHI agar for three days at 37°C. All stocks were kept at -20°C until used.

4.2.1.1 Isolation of single species from VSL#3

For single species isolation, 10 µl of VSL#3 \(1 \times 10^8\) cfu/ml livestock concentration was grown on Brain heart infusion (BHI) agar plate and incubated at either 37°C and 42°C incubator for 24 hours. To growth bifidobacterum species, Bifido selective medium (BSM) were used (Sigma, Cat no 88517 for agar and 90273 for broth) and incubated anaerobically for 3 to 5 days. Single bacterial colonies were picked based on different visual morphology such as size, form, elevation, margin of the whole
colony, colour, surface texture, density, and consistency of the colony. Bacterial stocks from individual colonies were produced by growing each picked colony in 10 ml BHI broth at 37°C in a shaking incubator (200 rpm) overnight, in a 50 ml loosen-lid falcon tube.

4.2.1.2 Verification of the isolation of the single species from VSL#3

To verify that a single species had been isolated from the poly-biotic VSL#3 each stock was plated on BHI agar medium plate for 24 hours at 37 °C. Colonies were screened for uniform morphology and one single colony was picked and sequenced. A colony was added to PCR master mix contains 2x BioMix (Bioline, cat no Bio-25006) for 15 µl, 1 µl of 16S Universal 1492 and 27f Primer forward and reverse of CGGTTACCTTGTTACGACTT and AGAGTTGATCMTGGCTCAG, and added distilled H2O until 30 µl. The thermocycling program consist of 94°C for 10 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute 30 second, all for 35 cycles, followed by 75°C for 10 minutes. The PCR product (2µl) was run on a 1% agarose gel electrophoresis, using GelRed® (Biomix, cat no 41003-1) 2 µl as intercalator, and 1 µl ladder (Invitrogen, Cat no. 15628-019). To verify the amplification of the gene target and the DNA quality and quantity, the PCR product was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and/or Qubit 2.0 Fluorometer (Invitrogen). Additionally, ratio optical density (OD) of 260/280 and 260/230 were read to assess protein and solvent contamination.
### Table 4.2 List of specific primers used to identified VSL#3 single species

<table>
<thead>
<tr>
<th>Species primers</th>
<th>Forward bp target</th>
<th>Length</th>
<th>TM</th>
<th>GC%</th>
<th>Self comp.</th>
<th>Self cmpl 3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strep. thermophilus (Sp.1)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>GCTCACCTAGGGCACGATAC</td>
<td>113</td>
<td>20</td>
<td>60.04</td>
<td>60</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>CCCATTGCGGGAAGATTCCCT</td>
<td></td>
<td>20</td>
<td>60.11</td>
<td>55</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><strong>B. breve Y8</strong></td>
<td></td>
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</tr>
<tr>
<td>R</td>
<td>GAAGGAGAAATCAAACCCCCGTC</td>
<td></td>
<td>23</td>
<td>62.73</td>
<td>52.17</td>
<td>3</td>
<td>3</td>
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<tr>
<td><strong>B. longum</strong></td>
<td></td>
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<tr>
<td>R</td>
<td>GGGAAAGCGGTATCCTCAAGA</td>
<td></td>
<td>20</td>
<td>58.4</td>
<td>50</td>
<td>4</td>
<td>2</td>
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<tr>
<td><strong>L. delbrueckii</strong></td>
<td></td>
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</tr>
<tr>
<td>F</td>
<td>CGACCAAATTGATCGTCTC</td>
<td>231</td>
<td>20</td>
<td>62.1</td>
<td></td>
<td></td>
<td>Seq results, home-made</td>
</tr>
<tr>
<td>R</td>
<td>ATGGTCTTGCCCTGCAAT</td>
<td></td>
<td>20</td>
<td>63.3</td>
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<tr>
<td><strong>L. plantarum</strong></td>
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<tr>
<td>F</td>
<td>GCAGGCTCAAATCCGACTG</td>
<td>217</td>
<td>20</td>
<td>59.97</td>
<td>55</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>R</td>
<td>GCAGGCTCAAATCCGACTG</td>
<td></td>
<td>20</td>
<td>59.23</td>
<td>60</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>16S RNA 1492 and 27 f</strong></td>
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<tr>
<td>F</td>
<td>CGTTACCTGGTACGACTT</td>
<td>1500</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>AGAGTTGATCMTGGCTCAG</td>
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</tbody>
</table>
The rest of the PCR product then purified using QIA quick purification kit (QIAGEN, Cat no. 28104) according to the manufacturer's instruction, then 50 ng/ml of the purified product along with 10 µl 16S reverse and forward primers send for sequencing to Beckman Coulter Genomics (BeckmanGenomics.com). The sequencing read then identified using BioEdit sequence alignment editor and compared to the reference BLAST database library (http://blast.ncbi.nlm.nih.gov).

To try to understand more about this probiotic, the live VSL#3 was grown on BHI/BSM medium agar plates at 370C and 420C environment for 24 hours to 5 days to growth the anaerobe bacteria. Individual bacterial colonies were picked based on different visual morphology of size, form, elevation, margin, colour, surface texture, density, and consistency of the colony colour, and size. Individual colonies were then grown in BHI/BSM broth overnight followed by plating on a BHI/BSM agar medium plate.

16S sequencing is a method used to identify individual bacterial species. It is based on 16S rDNA gene which forms part of the smaller subunit of ribosomes in a prokaryotic bacterium. This specific gene is highly conserve within living organisms from the same genus and species, but differs greatly between species.

4.2.2 Cell culture stimulation

THP-1 is a human leukemia monocytic cell line, which is commonly used to investigate macrophage/monocyte functions in human. This cells line were cultured in Falcon tissue culture flasks 750 ml (CORNING) and supplemented with RPMI-1640 plus Glutamax medium (Gibco, Cat no. 61870), 10% FBS (Sigma-Aldrich, Cat no. F9665), 20 mM HEPES (Sigma-Aldrich, Cat no. H0887-100ML), 100 U/ml Penicillin/Streptomycin (Life Tech, Cat no. 15140122), 50 µM 2-mercaptoethanol (Invitrogen, 21985023) and grown at 5% CO2 with air present at 370C.
Cells were stimulated with either TNF-α (10 ng/ml) (GIBCO, Cat no. PHC3105), heat killed \textit{E.coli} (HkEc) NCTC 10418 (gift from Andre Ribeiro) at microorganism of infection (Moi) 20, or lipopolysaccharide (LPS) (Enzo life science, Cat no. ALX-581-009-L001) 200 ng/ml for 24 hours. The effects of VSL#3 and single species (12.5–200 Moi) on the THP-1 cells either in isolation or in combination with TNF-α, HKEc, and LPS were investigated.

4.2.3 MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a colorimetric metabolic activity indicator used in cell viability assays. MTT 0.05% (Sigma M2128) in PBS. Cells were cultured in 96-wells plate within 100 μL of the medium under different conditions and time frames depending on the experimental protocol. The viability of the cells at the end of the protocol was determined by adding 30% of total volume of MTT (30 μL) to the wells and incubating in 5% CO2 at 37°C condition for at least 4 hours. MTT is converted to formazan, a dark blue water-insoluble material through the reducing activity of mitochondrial dehydrogenases in live cells. The plate was then centrifuged at 8000 g for 5 minutes and supernatant carefully removed. Formazan is solubilized through the addition of 100 ul lysis solution (900 mL isopropanol, 47 ml dH₂O, 50 ml 10% sodium dodecyl sulphate (SDS) and 3 mL concentrated HCl). The solution was pipetted multiple times until the formazan crystals dissolve. Plates were read on a FLUOstar Omega microplate reader (BMG LABTECH) at OD₅₆₃ nm.

4.2.4 Species-specific primers designed

For this primers design, full receptor DNA sequences were obtained either from GenBank (https://www.ncbi.nlm.nih.gov/genbank) or according to my previous
DNA sequencing results. DNA sequences of target genes were analysed using the Primer3plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The PCR products then purified using Monarch® DNA gel extraction kit (NEB, cat no. T1020S) according to manufacturer’s instruction. The purified PCR product then prepared for sequences using Mix2 Swq Kit (https://www.eurofinsgenomics.eu/en/custom-dna-sequencing/eurofins-services/mix2seq-kits/) before sent to the same company for sequencing. The sequencing read then identify using BioEdit sequence alignment editor and compared to the reference BLAST database library (http://blast.ncbi.nlm.nih.gov).

4.2.5 Saliva sampling

Two healthy young gentlemen aged 37 and 43 years old kindly volunteering themselves to give their saliva before and after they consumed their first ever VSL#3 multi-biotic (Informed consent following the informed consent for healthy control under Research etich committee (REC) reference no.10/HO806/115 with IRAS project ID of 28159. Five ml saliva samples collected before VSL#3 consumption and on 1, 2, 4, 6, 12, and 24 hours afterward on 50 ml falcon tubes contained saliva buffer (as written on Chapter 2 section 2.5.3). Saliva DNA was extracted using PurElute™ bacterial genomic kit from EdgeBio (cat No. 85171) and qPCR performed using specific primers for S. thermophilus, L. plantarum, B. breve, B. longum, and L. delbruecksi subspecies bulgaris, 16S 1492 and 27f universal primers were used to normilize the results to bacterial numbers within saliva.

4.3 Statistic

All data are presented as mean ± SEM using GraphPad Prism 6.0 (GraphPad Software, Inc). When analysis involved more than two groups, statistical significance
was calculated using one-way ANOVA with Dunnett's multiple comparison test with a 
single pooled variance or mention as indicated.

4. 4 Results

4.4.1 Single species isolation from VSL#3

From the VSL#3 BHI agar plate, 12 morphologically different bacterial colonies 
were picked, grown and underwent 16S sequencing. The twelve colonies sent for 16S 
sequencing resulted in the identification of 6 different bacteria species. These were 
*Streptococcus thermophilus*, *Lactobacillus paracasei*, *Lactobacillus casei*, 
*Lactobacillus plantarum*, *Micrococcus luteus strain*, *Bacillus Species* and 
*Lactobacillus acidophilus* was also found in a second screen from the aerobic growth. 
I was able to isolate four of the eight VSL#3 bacteria which were taxonomically 
assigned to a species based on their 16S rRNA gene sequences and identified to the 
species level. The identified species were *S. thermophilus*, *L. paracasei* / *L. casei*, 
*L. plantarum*, and *L. acidophilus*. *L. paracasei* and *L. casei* were actually similar and 
it has been suggested that they should be classified as one species.\(^{(225)}\) *M. luteus 
strain* and *Bacillus sp* were probably contaminants from the lab as they are both 
commonly found in dust.\(^{(226)}\) The use of specific medium for *Bifidobacterium* species 
did not result in the isolation of the expected microorganism. There were colonies on 
the media which put in anaerobe chamber, however the sequencing results identified 
them as *L. plantarum* and *S. thermophilus*. Both microorganisms are facultative 
bacteria which generating adenosine triphosphate (ATP) by aerobic respiration in the 
presence of oxygen, however in anaerobic conditions these species can switch to 
fermentation. The 42\(^{\circ}\)C cultivation resulted in only the isolation of *L. acidophilus*. 
Three species could not be isolate from VSL#3 using this method. The first one is
Lactobacillus delbrueksii subspecies bulgaricus, which usually needs a high temperature to growth (40°C-44°C).\(^{(227)}\) The other two are Bifidobacterium brevis, B. longus, and B. infantis which could not be isolated even after cultivation under anaerobic conditions on specific media for bifidobacterium.\(^{(228)}\) One reason for this could be technical where all anaerobe bacteria contained in the sachets have been killed due to oxygen exposure when stocks were prepared.

Morphologically different isolates from VSL#3 demonstrated S. thermophilus which characterised by colony size of ~1 mm, circular in form, convex, entire edge colonies, white, glistening surface, opaque, and butyrous consistency. This strain has identity score of 100 % relatedness to S. thermophilus CNRZ1066 (NCBI accession no: NC_006449.1). L. paracasei colonies phenotypically showed less than 1 mm size, circular, convex, entire edge colonies, white, glistening surface, and butyrous consistency and identity score of 100 % relatedness to L. paracasei strain ATM5 (NCBI accession no: KR919617.1). The closely related group of L. casei demonstrate bigger size, similar circular form, convex, entire edge colonies, white, glistening surface, and butyrous consistency) with identity score of 100 % relatedness to L. casei ATCC 334 (NCBI accession no: NC_008526.1). L. plantarum identified morphologically by colonies less than 1 mm in size, raised, entire edge colonies (smooth, regular), creamy white, dull surface, and butyrous in consistency and had identity score of 100 % relatedness to L. plantarum WCFS1 (NCBI accession no: NC_004567.2). L. acidophilus characterised by very small diameter-pin point colonies, circular, raised, entire edge colonies, whitish, glistening surface, transparent, and butyrous consistency with identity score of 98.54 % relatedness to L. acidophilus NCFM (NCBI accession no: NC_006814.3).
4.4.2 Identification of VSL#3 single species using specific primers

Having isolated and verified 4 out of the 8 species in VSL#3, I then tried to identify the rest using specific primers for the individual species. After extracting bacterial DNA from VSL#3 and performing a PCR amplification using the primers listed in Table 4.2 methods section. The PCR products were purified and sent for DNA sequencing. I was able to identify *S. thermophilus*, *B. breve*, *B. longum*, and *L. delbruecksi subspecies bulgaricus*.

4.4.3 Potential adaptability of multi-probiotic VSL#3 species in the oral environment

To understand more about the potential of poly-biotic VSL#3 to integrate in and adapt to the oral environment, two different methods were used. Firstly, saliva DNA was extracted from 20 samples taken from healthy volunteers for 16S bacterial gene PCR amplification, purification, and sequencing. Hypervariable region of V5 to V7 were amplified using illumina MiSeq platform. The 16S sequencing results were then compared with published known commercial probiotic species. The results were also equated with a previous study which sequenced a Jewish population cohort of 191 by our group. (229) Secondly, saliva samples before and after VSL#3 probiotic consumption were collected from two healthy volunteers.
Total species in oral cavity of 191 participants in Jewish population

- Total species: 845
- VSL#3: 5 (45%)
- Others probiotic: 6 (55%)
- Species: 11 (1.3%)

Figure 4.2 Pie chart depict number of probiotic species found in the saliva from two cohort compared with the total number of species. A. the Jewish population (n=191) were found to have 845 phylotypes including 11 that are claimed to be probiotic species in their saliva. Four of the saliva resident species were the same species as found in VSL#3. B. 1134 phylotypes found with 8 of these species claimed to have benefit to the host. Four probiotic species were the same species as found in the VSL#3.

Total species in oral cavity of 20 healthy control participants

- Total species: 1134
- Claimed as probiotic: 8 (0.8%)
- VSL#3: 4 (50%)
- Others probiotic: 4 (50%)
- Species: 1134
It is interesting to note that heterogenous and homogenous cohort demonstrated difference in the number of probiotic VSL#3 species detected in saliva of two cohort group. These differences might be due to the different populations used for each study. Our groups publication regarding the role of genetics on the saliva microbiome demonstrated that the dominant factor contributing to the diversity in salivary microbiome composition is the environmentally rather than genetic. Other reason might be the number of subjects participated in the experiment. Jewish cohort has 191 subjects which statistically means more possibility to found specific targeted species than only 20 random samples we recruited. However, the contrary applied for species diversity where 20 subjects with different ethnic background demonstrated more diverse species than more homogenous AZ Jewish group.

The last contributed factor might be different kit used for 16S sequencing. Our previous 16S sequencing used kit for Ilumina MiSeq system with quality control, normalisation, pooling and sequencing on a single MiSeq v2 500 cycle catridge and 250 bp paired end and commonly targeting hypervariable region of V3 to v4. This system then advanced with new 16S kit from Swift Bioscience (https://swiftbiosci.com/swift-amplicon-16s-its-panel). This kit provides coverage of all of the hypervariable region (V1 to V9) of the 16S gene and the fungal ITS1 and ITS2 (ribosomal_ Internal transcribed spacer region) amplicon, sequenced on the II, however this does on Ilumina MiSeq platform. ITS1 and ITS2 are DNA metabarcoding marker that characterize the diversity and composition of fungal communities.
Figure 4. 3. Diagram Venn of individual species from two different populations. Jewish population (n=191) has less diverse phylotype with only 283 different species from the ethnic diverse population (n=20) which has 572 species. Both population shared the same 562 species. (Species listed on appendix 2)

Figure 4. 4. Enumeration of total VSL#3 species composition before and after multi-probiotic administered. The graph showed that VSL#3species i.e *Streptococcus thermophilus*, *B. breve*, *B. longum*, *L delbrueckii sub bulgaricus*, and *L. plantarum* could attach to oral environment in the first hour after VSL#3 consumption. This number then reduced as the time increases until 24 hours, but tend to increase up to 72 hours for *S. thermophilus* and *L. delbrueckii sub bulgaricus*. 
To understand more about adaptability of individual strain of VSL#3 on the oral environment, the in vivo attachment assay was performed. As figure 4.7 demonstrated, *S. thermophiles* and *L. delbrueckii sub. Bulgaricus* increased by almost half in the first 2 hours. The single species could not be attached longer for the rest of the period. The main reason for this might be because in this assay, the volunteer allowed to consume their daily meal and snack which potentially flushing down the single probiotic. Secondly, probiotic package including VSL#3, although was not clearly mention about it, commonly have a microencapsulation which help to preserve contained microorganism to safely go through the target of intestinal. Due to this reason, it is assumed that the microencapsulated form might prevent the bacteria to attach to the mouth epithelium.

### 4.4.4 Single species effects on THP-1 viability

As a part of the characterization of the four single isolates: *S. thermophilus*, *L. paracasei* / *L. casei*, and *L. plantarum* an MTT viability assay was perform using THP-1 cells. This cell-based assay is used to determine if an agent has an effect on cell proliferation and/or direct cytotoxic effects leading to cell death. HkEc at 20 Microorganism of Infection (Moi), which induces THP-1 activation and inhibits cell proliferation was used as a positive control.\(^{(232)}\) To observe the effects on THP-1 cells, single isolates or VSL#3 were used at a single dose of 50 Moi or in a dose range (12.5 to 100 Moi) to stimulate THP-1 cell. Live, heat killed, or formaldehyde fixed VSL#3 had no effect on THP-1 cells viability (Figure 4.7.A and 4.7.B) or proliferation (Figure 4.7.C) up to 100 Moi. This is in contrast to the effect of stimulation by HkEc which resulted in a potent loss in THP-1 cell proliferation (Figure 4.7.A and 4.7B). In addition, the four single isolates showed no effect on THP-1 cell viability nor proliferation (Figure 4.7D) for VSL#3 and in contrast to HkEc.
Figure 4.5 THP-1 treatment with different stimulants. A. THP-1 cells viability after stimulation with VSL#3 or VSL#3 with HkEc for 24 hours incubation with different coefficient of infection. B. Analysis of cells viability on different stimulant, at 25 coi. C. Cells viability after stimulation with VSL#3, VSL#3 with HkEc, and single species (D) for different time course. (All data represent in biological duplicate and use 25 moi of VSL#3 and 20 moi of HkEc unless stated different ns = p > 0.05  *= p ≤ 0.05 ** = p ≤ 0.01 *** = p ≤ 0.001.
Figure 4.6 Cell attachment assay (A) and cell viability assay (B) using MTT. A. THP-1 cells stimulated with either HkEc (20 coi), LPS (200ng/ml), TNF (10ng/ml), PMA (200ng/ml), VSLHk, VSLFf, VSLL, or single isolate (all in 50 coi) for 24 hours. *= p ≤ 0.05 ** = p ≤ 0.01 *** = p ≤ 0.001.
Stimulation of THP-1 cells results in phenotypic alteration and the switch from a suspension cell (monocyte-like) to an adherent (macrophage) transition.\(^{(233, 234)}\) The level of cellular differentiation and attachment provides a basic readout of immune activity of a stimulant. PMA induced highest adhesion activity with THP-1 cells, followed by HkEc, LPS, and TNF (Figure 4.8.A). In contrast, both VSLHk and VSLL were much less potent at inducing THP-1 cells to differentiate into mature macrophages. Furthermore, neither VSLFf nor four single isolates were able to increase adhesion levels above that of unstimulated THP-1 cells (Figure 4.8.A). When THP-1 cells were stimulated for 24 hours in combination with HkEc, VSL#3 demonstrated a potential to inhibit HkEc effect by increasing cell viability in contrast with HkEc stimulation alone (Figure 4.8.B).

### 4.4.5 The effects of single species of VSL#3 on cytokine secretion

A major response of THP-1 cells to immune activation is the synthesis and secretion of cytokines and chemokines. Therefore as a part of the characterization of the single species and VSL#3 I quantified the levels of cytokine release by THP1 cells after stimulation. Due to my research being focused in OLP I studied two pro-inflammatory cytokines CXCL10 and IL-6 which have been associated with disease severity. IP10 is a chemoattractant chemokine that has been shown to be elevated in OLP.\(^{(24, 31)}\) Ichimura et al previously showed that oral epithelium and lamina propria of OLP patients express high levels of CXCR3 which is the cellular receptor for CXCL10 and orchestrates the signalling for T cell infiltration in to lesional areas.\(^{(24)}\) While IL-6 is recognized as an important protein for OLP and has been suggested as an indicator of OLP disease activity.

VSL#3 has the ability to increase the secretion of CXCL-10 in contrast to the single isolates and unstimulated cells (Figure 4.9.A). The ability of LPS to stimulate
THP-1 cells and induce CXCL10 secretions is about six times greater than VSL#3. The co-stimulation of THP-1 cells with LPS and VSL#3 resulted in the loss in CXCL10 secretion compared to LPS alone. The same inhibitory effects on LPS stimulation were found with all four single species and irrespective if VSL#3 was live, heat killed or fixed (Figure 4.9.B). In addition to LPS, VSL#3 also demonstrated the ability to partial reduce the release of CXCL10 upon TNF stimulation (Figure 4.9.C). All four single isolate could totally block CXCL10 secretion upon TNF stimulation (Figure 4.9.C).

VSL#3 alone could induce significantly more IL-6 secretion compared to unstimulated cells and cells stimulated with the single isolates (Figure 4.10.A). Additionally, LPS stimulation resulted in an increase in IL-6 secretion, which could be partially inhibited by both single isolates and VSL#3 (Figure 4.10.B). VSL#3 was more potent than the single species at inhibiting IL-6 secretion after LPS stimulation. THP-1 cells stimulated with TNF demonstrated a similar pattern of IL-6 secretion as seen with LPS (Figure 4.10.C). The results for IL-6 differ from what were found for CXCL10 where LPS effect inhibit by VSL#3 resulted in the loss in CXCL10 secretion compared to LPS alone. Difference responds of IL-6 and CXCL10 suggesting that VSL#3 and probiotic bacteria may have different effects on different cytokines.
Figure 4. 7 CXCL10 protein level with VSL#3 stimulation A. Before combined with LPS , B. after stimulation of VSL#3 with LPS 200 ng/ml, C. and TNF 10 ng/ml. Data from two assay of four replicates each.
Figure 4.8 IL-6 protein level with VSL#3A. without stimulation B. after stimulation of VSL#3 with LPS 200 ng/ml, and TNF 10 ng/ml (B). Data from four replicate.
4.3.7 The effects of VSL#3 on cytokine gene expression

The inhibitor effects of probiotics on cytokine secretion can work by blocking bacterial/immune receptor activation, inhibit the downstream signalling, block gene induction or influence protein synthesis and secretion. To determine if probiotic exposure works at the level of protein synthesis and secretion level of gene induction was determined. VSL#3 probiotic and single isolates in inhibit the induction of both CXCL10 and IL-6 at the mRNA transcriptional level 24 hours after stimulation (Figure 4.11.A, 4.11.B, 4.11.C, and 4.11.D). These results do not correlate exactly with secretion levels and further work will be needed to determine the mRNA levels at different time points after stimulation. The inhibition of cytokine gene activation and secretion could have important implications for the probiotic VSL#3 being used to treat disease which are associated with an elevation in these molecules. VSL#3 might reduce disease activity within the OLP lesion based on recent findings that reported elevated levels of CXCL10 and CXCR3R in OLP lesion. (78)
Figure 4.9 CXCL10 and IL-6 mRNA levels after stimulation with VSL#3, SI, and/or LPS for 24 hours at 25 Moi A. CXCL10 level after stimulation with VSL#3 and/or LPS. B. CXCL10 level with single isolate stimulation and/or LPS. C. IL-6 level after stimulation with VSL#3 and/or LPS. D. IL-6 level after stimulation with single isolate and/or LPS. The results represent the mean (±SEM) three biological replicates.
4.4. Discussion

It has been long known that probiotics have the ability to convey beneficial effects to its host. These effects have been studied intensively with a varying level of sophistication. The majority of microorganisms which have been claimed to be probiotic either in isolation or as a more complex mixture have been well characterised and used in numerous trials. However, none of these studies characterise single species which originate from the direct marketed product. - Doullard et al (2018) have characterised mechanisms of action of each species in multi-probiotic VSL#3. In the process of sequencing and characterisation of the 8 species contain in this cocktails probiotic, the author used the DNA mixture of the microorganisms provided by the associated manufacturer instead culture it directly from the original marketed product. (222)

VSL#3 is reported to contain eight different bacteria Lactobacillus acidophilus (BA05), Lactobacillus delbrueckii subsp. Bulgaricus (BD08) (reclassified as lactobacillus helveticus_product label information), Lactobacillus paracasei (BP07), Lactobacillus plantarum (BP06), Bifidobacterium longum (BL03), Bifidobacterium infantis (BI04) (BL03 and BI04 reclassified as B. animalis subsp. lactis_product label information), Bifidobacterium breve (BB02), and Streptococcus thermophiles (BT01). Apart from blended bacteria, VSL#3 also contains maltose (sweetener) and silicone oxide (anti-craking agent).

To further understanding the single species role and predicting efficacy of the single strain, the live VSL#3 was grown on BHI medium for 24 hours. Different morphologically colonies then picked up and sent for 16S sequencing. Out of 8 species I found Streptococcus thermophilus, Lactobacillus paracasei, Lactobacillus
casei, Lactobacillus plantarum, and in the next culture, Lactobacillus acidophilus. It is interesting that in this culture we found two taxonomically related strains L. paracasei and L. casei while the investigational product label only mentions L. paracasei BP07. It has been long debated about the taxonomic position of L. casei and its subspecies among the microbiologists.\(^{225}\) Classification of species and subspecies are currently based either on DNA homology data or sequencing signature from the 16S rRNA. Although, this Lactobacillus group can be readily distinguished from other members of the Lactobacillus genus by fermentation profiles, the classification is not yet stable.\(^{225, 235}\) According to Mori (1997) sequencing of both L. paracasei and L. casei ATCC 334 were actually similar, and based on other reports that identified high DNA-DNA relatedness between L. paracasei NCDO 151\(^T\) and L. casei ATCC 334 it has been suggested that both strain should be classified as one species.\(^{225}\) In this study, we treated both clones as a different strain to see whether they could trigger the same or different immune responses.

There were four other strains which are purported to be contained in VSL#3 that have so far not been found, Lactobacillus delbrueksii subspecies bulgaricus which needs a high temperature to growth (40\(^0\)C - 44\(^0\)C).\(^{227}\) The other three are Bifidobacterium brevis, B. longus, and B. infantis which could not isolated even after cultivation under anaerobe chamber on specific media for bifidobacterium.\(^{228}\) One reason for this unsuccessful attempt is technical faulty where all anaerobe bacteria contained in the sachets have been dead due to oxygen exposure when stock was prepared. Secondly, it could be related to wrong colony chosen, and third the 16S universal primers of 1492 and 27f were not specific enough to differentiate targeted strain as there are more than 125,000 different universal 16S rRNA targeting different conserve region.
To identify other species contained in VSL#3 species-specific primers were designated and used as a template to identified whole DNA sequencing extracted from VSL#3. This approach identified the additional non-culturable 5 single species (Str. thermophilus, L. plantarum, B. breve, B. longum, and L. dulbreuckii subspecies bulgaricus). In total, both analysis identified 7 out of 8 strains, with remaining B infantis unidentified. The B. infantis and B. longum recently reclassified as B. animalis subs lactis strain due to only few SNPs (single-nucleotide polymorphisms) and InDels (Insertion/deletion polymorphisms) different between the two, indicating that both stains share a very recent and clonal ancestor.\textsuperscript{(222)}
Table 4.3. Species claimed to be members of the probiotic group of bacteria found in the saliva* from 191 Jewish cohort

<table>
<thead>
<tr>
<th>No.</th>
<th>Known Probiotic species*</th>
<th>No of indv with sp</th>
<th>Lowest</th>
<th>Highest</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactobacillus rhamnosus</td>
<td>16 (8.4)</td>
<td>0.000340377</td>
<td>0.0270354</td>
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<tr>
<td>2</td>
<td>Lactobacillus acidophilus</td>
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<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Lactobacillus plantarum</td>
<td>6 (3.1%)</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
<td>Lactobacillus delbrueckii subsp bulgaricus</td>
<td>7 (3.7%)</td>
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<tr>
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<td>Lactobacillus reuteri</td>
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</tr>
<tr>
<td>10</td>
<td>Bifidobacterium infantis</td>
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<td>-</td>
</tr>
<tr>
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<td>Bifidobacterium animalis subs lactis</td>
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<td>-</td>
</tr>
<tr>
<td>26</td>
<td>Escherichia coli Nissle 1917</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Red mark indicating the same species contain in VSL#3

The lowest and the highest value represent the fraction of that species out of the total number of reads (with 1 as a maximal value).
Table 4.4. Species claimed to be members of the probiotic group of bacteria found in the saliva * from 20 random healthy controls

<table>
<thead>
<tr>
<th>No.</th>
<th>Known Probiotic species*</th>
<th>No of indv with sp</th>
<th>Lowest</th>
<th>Highest</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>Lactobacillus plantarum</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>Lactobacillus casei</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td><em>Lactobacillus delbrueckii subsp bulgaricus</em></td>
<td>1 (5%)</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td><em>Lactobacillus brevis</em></td>
<td>0</td>
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<td>-</td>
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<td><em>Lactobacillus johnsonii</em></td>
<td>19 (95%)</td>
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<td>1 (5%)</td>
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<td>0.026</td>
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<td>3 (15%)</td>
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<td>10</td>
<td><em>Bifidobacterium infantis</em></td>
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<td>-</td>
</tr>
<tr>
<td>11</td>
<td><em>Bifidobacterium animalis subsp lactis</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td><em>Bifidobacterium bifidum</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td><em>Bifidobacterium longum</em></td>
<td>3 (15%)</td>
<td>0.001</td>
<td>0.282</td>
</tr>
<tr>
<td>14</td>
<td><em>Bifidobacterium breve</em></td>
<td>3 (15%)</td>
<td>0</td>
<td>0.017</td>
</tr>
<tr>
<td>15</td>
<td><em>Bifidobacterium lactis subsp. Lactis</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td><em>Saccharomyces boulardi</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td><em>Enterococcus durans</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td><em>Enterococcus faecium</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td><em>Streptococcus thermophilus</em></td>
<td>20 (100%)</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>20</td>
<td><em>Streptococcus salivarius</em></td>
<td>3 (15%)</td>
<td>0</td>
<td>0.002</td>
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<tr>
<td>21</td>
<td><em>Pedicoccus acidilactici</em></td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td><em>Leuconostoc mesenteroides B7</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td><em>Bacillus coagulans</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td><em>Bacillus subtilis</em></td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td><em>Bacillus cereus</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td><em>Escherichia coli Nissle 1917</em></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


* Red mark indicating the same species contain in VSL#3
Duillard et al. (2018) who isolated the single species from VSL#3 using extracted DNA and identified 6 out of 8 strains. *S. thermophilus* (BT01), *L. acidophilus* (BA05), *L. plantarum* (BP06), *L. paracasei* (BP07), *B. longum* (BL03), and *B. infantis* (BI04) based on their 16S rRNA gene sequences to the species level. The author then identified the other two strains, *Lactobacillus delbrueckii* subsp. *Bulgariicus* (BD08) and *Bifidobacterium breve* (BB02) using proteomes comparison. The ability to detect the species by DNA sequencing does not provide any evidence that they are still viable within the mixture and my inability to grow some of the strains could be due to a loss in viability during the freeze drying process and storage. Alternatively, it could be since I have not used the ideal conditions. Further work will be needed to determine the exact viable constituents within VSL#3. This is currently an important consideration judging that the VSL#3 is now under dispute with another company which claimed that currently marketed VSL#3 (referred as new formulation) contained different species formulation and less viable bacterial cell numbers compared to the old formulation (currently marketed under Vivomixx, Visbiome of Desimone formulation).

Mora et al., in 2019 publication performed shotgun metagenomics by deep ilumina sequencing for the DNA isolated from the commercial VSL#3 aimed to confirm strain-identity safety and composition. The group also performed single-cell analysis to evaluate the cell viability, and used β-galactosidase and urease activity as marker to monitor the reproducibility of the production process. Furthermore, they were characterized the same lot in detail by a metaproteomic approach for which a robust protein extraction protocol was combined with advanced mass spectrometry. Interestingly, this group reported live cells of approximately $3.8-3.9 \times 10^{11}$ which is higher on the number of live cells declared in the label. The dead cells were between
8 to 10% of overall population in both lots, emphasizing an optimal biomass production process.

As a part of our characterization of the single species contained within VSL#3, 16S sequencing of saliva from 20 randomly recruited-healthy control volunteer was performed and the results compared with our previously published study results (Table 4.3, 4.4). Our results demonstrated that normal oral microbiota composition from both population contain claimed-benefit microorganism, some of them were might be the same strain of VSL#3 probiotic mixture. These suggest that VSL#3 individual strain has high potential adaptability with oral environment and could be used oral environment as the targeting-site.

It is also interesting to note that the ethnic diversity population of 20 healthy participants uncovered more species diversity compare to the 191 Jewish population. Our previous group publication suggesting that the environment rather than host genetics is the dominant factor affecting the composition of the salivary microbiome in closely blood related individuals. This study comparison result in agreement with those suggestion where the homogeneity population which assumedly have similar lifestyle behaviour dictated less diversity of taxa on those group. Conversely, in a more heterogenic ethnic diversity, the daily culture which led to more assorted way of life also generating more richness of taxa on salivary microbiome.

Furthermore, we also conducted single species identification from 2 healthy control volunteer saliva before and after VSL#3 administration. This study demonstrated to us that an hour after intake of the poly-biotic microorganism, both *S. thermophillus* and *L. dulbrueckii sub. Bulgaricus* have increased and gradually reduced until 24 hours before it began to again increase until 72 hours. The reason for this increasing might be associated with other diary-contain food which is potential
to contain the benefit microorganism or might be associated with the bacterial proliferation. Additionally, the *L. plantarum* which could not detect from both volunteer before the intake, also displayed trace of increased up to two hours. The possibility of the VSL#3 single species to adhered and interacted in the oral environment supported by this experiment and the two microbiome experiments performed in our group. Some publications also reporting the series of ability of the anaerobe Bifidobacterium to attach and interact in the oral environment.\(^{(237, 238)}\)

To further investigate whether the THP-1 cells responses were due to an active interaction with live microorganisms, the VSL#3 was treated in different ways resulting in three different stocks: live, heat killed, and formaldehyde treated. These were tested over a dose range of 12.5 to 100 Moi for the VSL#3 mixture and at a single dose of 50 Moi for the single species. The three different VSL#3 stocks had no effect on THP-1 cells viability or proliferation. This suggested that the probiotic effects are likely mediated through a cell surface protein rather than active metabolite secretion. Similar results have previously been reported by Hoermannsperger (2009) who demonstrated inhibition of CXCL10 secretion by heat-inactivated and formaldehyde fixed VSL#3.\(^{(111)}\) We suggest that

The cell adhesion assay was performed to observe effect of VSL#3 on THP-1 cell stimulation and activation. Upon THP-1 activation the cell undergoes cell cycle arrest and adheres. This morphology and activation results in the maturation of the THP-1 cells into a pro-inflammatory macrophage and the secretion of cytokines. This assay was performed by stimulating THP-1 cells either with VSL#3, single isolate, LPS, HkEc, or phorbol-12-myristate-13-acetate (PMA) as a positive control for 24 hours. PMA is an effective differentiation agent that drives the generation of mature THP-1 monocyte-derived macrophages. The THP-1 monocyte will respond upon
stimulation by differentiated into macrophage which results in cell spreading and elongation, allowing the cells to attach on the plate.

Furthermore our results also demonstrated potential of both VSL#3 in combination and single species of *S. thermophilus*, *L. paracasei casei*, and *L. plantarum* in inhibiting LPS induced CXCL10 regardless of the live, heat inactivated, or formalin fixed status. In addition to LPS, VSL#3 in combination and in four single species also demonstrated the ability to partially reduce the release of CXCL10 upon TNF stimulation.

These results highlighting the potential role in attenuating the innate immune response by VSL#3 as one of important mechanism which might explain their benefit in treating some oral disease. The VSL#3 inhibit immune response activation mostly via reducing the extent of signalling pathway via Toll like receptor 4 (TLR4) and Tumor Necrosis Factor Receptor (TNFR) with unknown receptors yet. TLR4 and TNFR activation led to release of Nf-KB cytokines dependent such as CXCL10 which, in our previous chapter, increased and might play critical role in diseases such as OLP. Although, TLR4 mainly expressed on the professional immune cells like macrophages, but there is huge evidence of capability of oral epithelial cells in expressing these ligands. (85, 182, 239) Key evidence linking TLR4 with the pathogenesis of OLP supported by the enhanced expression of TLR4 in the whole unstimulated saliva (UWS), PBMC, and OLP lesion compared to healthy control. (31, 45, 48, 85) These evidences might be supported by serial publication regarding dysbiosis of oral cavity in the OLP patient. (33, 45, 48, 85, 100) as Janardham et al along with other authors found that enhanced Gram-negative bacteria in the OLP oral environment might be the source of significant increase in TLR4. (34, 36, 48) Once oral epithelial TLR4 detected LPS on bacterial surface, it would leads to the recruitment of the accessory molecules
myeloid differentiation primary response (MYD88) or Toll/interleukin domain-containing adapter (TIRAP), and eventually leading to the induction of the transcription factors (NFkB) with a final outcome of hyper-secretion of NFkB-dependent cytokines including IL-6 and CXCL10.\(^{(240)}\) the over expression of those pro-inflammatory signalling then leading to mucosal injury due to massive infiltration of inflammatory cells. In this regard, we suggested that probiotic VSL#3 might benefit to ameliorate OLP inflammatory sign and symptoms.

Serial ability of the probiotic strain in blocking TLR4 and TNFR in different kind of diseases have been reported by some authors. Good et al, used intestinal tissue from human infant that were previously treated for necrotizing enterocolitis (NEC) to stimulate with either LPS or DNA of \(L.rhamnosus\)-pre-treated cells with LPS. Their results demonstrated capability of the benefit microorganism to deter TLR4 secretion.\(^{(241)}\) Kanmani et al, also demonstrated that benefit microorganism of Lactobacillus species (\(Weissella cibaria, lactobacillus sakei\) and \(L. plantarum\)) is a potent inhibitor for TLR4 in LPS-stimulated THP-1 cells.\(^{(242)}\) Furthermore, Xiong et al proved that mucus binding protein origin from \(L.acidophilus\) could downregulate TLR4-induced LPS stimulation on caco-2 and Raw 264-7 cell lines. Additionally, the same authors reported TNF-\(\alpha\) reduction after variety of benefit microorganism stimulation.\(^{(239, 241-243)}\)

Taken together, this study has provided us with suggestive treatment strategic approach for OLP using benefit poly-microorganism via inhibition of the pro-inflammatory immune response downstream of both TLR4 and TNFR.
CHAPTER 5
EFFECTS OF MULTI-BIOTIC VSL#3 ON ORAL EPITHELIAL IMMUNE RESPONSE

5.1 Introduction

The epithelium layer covering the oral mucosa has an important function as a barrier against chemicals, microbial molecules and physical challenges coming from the environment. The protective barrier is built up from a complex interaction between structural and functional proteins with the ability to counteract both external and endogenous challenges.

Several mechanisms are involved in the protective process, such as generation of an extracellular mucin layer, production of antimicrobial peptides and the formation of intracellular tight junctions which form a tight seal of epithelial layer. Additionally, the outer epithelial layer also functions as a controlling gate for ions and water-soluble solutes through the paracellular space.^(244)^

Intracellular tight junction integrity is a critical component of the protective function for the epithelium. Disruption to this integrity leads to decreased anti-microbial functions towards harmful antigens or pathogenic bacteria and can result in mucosal damage, more tolerant mucosal permeability, and overgrowth of bacteria leading to dysbiosis.^(245)^

The oral epithelial cells are also endowed with immune functions. In 2006, Sugawa et al found that oral epithelial cells have the capability to produce peptidoglycan recognition proteins, including toll-like receptors (TLR) and NOD-like receptors which respond to bacterial components.^(246)^ Additionally, oral epithelial cells have been shown to express TLRs.^(182, 239, 243)^ Ten TLRs have been identified in human which are localized either extracellularly (1,2,4,5,6 and 11) or intracellularly
in the endoplasmic reticulum and endosomes (3,7,8 and 9).\(^{(31)}\) TLRs recognise pathogen associated molecular patterns (PAMPs), alarmins or damage-associated molecular patterns (DAMPs). Receptor activation results in the generation of a signalling cascade which leads to NF-κB and/or interferon response factor-3 (IRF3) activation and pro-inflammatory gene transcription, such as IL-6, IL-10 and IL-12.\(^{(247)}\)

Inappropriate TLR responses either because of a genetic defect or over sensed alarmins or DAMPS generated from endogenous molecules from dying cells, or in some pathological conditions would stimulate the initiation or exacerbation of inflammatory and autoimmune diseases.\(^{(248)}\)

Alteration in TLRs expression has been reported in oral lichen planus (OLP), an autoimmune disease with unknown aetiology. This disease manifest in a variety of forms ranging from an asymptomatic reticular phenotype to painful erosive and ulcerative lesions. Domingus reported altered TLR3 and TLR7 activation as the cause of high TNF-α responsiveness in peripheral blood mononuclear (PBMC) cells from patients with lichen planus.\(^{(31)}\) An increase in TLR2 expression has been identified in lesion tissues and PBMC from OLP patients.\(^{(45)}\) While Siponen et al reported an elevation in TLR4 and TLR9 in oral epithelium from patients with OLP compared to health subjects.\(^{(85)}\)

Not all microbes are equal when it comes to immune activation and development of inflammation. The World Health Organisation in 2002 define probiotic as a live microorganism which when consume in adequate amount would convey benefit to the host.\(^{(188, 249, 250)}\) Figure 5.1 demonstrated general probiotic mechanism of action based on genus classification.\(^{(251)}\)
In dentistry, probiotics have been applied for some diseases. Jung, performed double blind, randomized, placebo-controlled trial using a probiotic combination of *Bifidobacterium longum*, *Lactobacillus lactis*, and *Enterococcus facium* for 7 weeks with a dose of 3 capsules 3 times a day and measured severity of oral mucositis induced by chemotherapy for patients with nasopharyngeal carcinoma. He concluded that probiotics significantly enhances the immune response of patients and

![Figure 5.1 Probiotic mechanism of actions. Shared mechanism of actions based on genus classification. (Courtesy of Sanders, M.E., et al. (2018). Shared mechanism among probiotic taxa: implication for general probiotic claims. Curr Opin Biotechnol 49: 207-216.)](image-url)
reduces the severity of oral mucositis via modulation of gut microbiota. In another study, Teughels reported that oral administration of *Lactobacillus reuteri* lozenges could be an useful adjunct to scaling and root planning in chronic periodontitis.\(^{150}\) Additionally, Erika G.S demonstrated that *petit-suisse* - fresh cheese, not matured, obtained by the coagulation of milk or without addition of other food substance-contained *Lactobacillus casei* which had the ability to decreased *Agreggatibacter actinomycetemcomitans* and maintain a low density of *Porphyromonas gingivalis* in children’s saliva.\(^{252}\) Both bacteria commonly found in oral cavity environment and associated with chronic periodontitis or gingivitis.\(^{253}\)

Figure 5.2 listed probiotic mechanism of actions in conveying benefit in oral cavity environment.\(^{250}\) according to Meurman, the hypothetical mechanism of the probiotic as this figure 5.2 demonstrated should be investigated for the prevention and treatment of dental and oral mucosal diseases.\(^{250}\) Based on this article and from observation that there are only very few studies addressing issues relating to the effects of probiotics on the oral environment or on oral epithelial cells. Using the commercially available poly-biotic VSL#3 which compromises 8 different probiotic species we investigated the anti-inflammatory potential on oral epithelial cells
5.2 Material and methods

5.2.1 VSL#3 stock preparation for cells stimulation

VSL#3 bacterial stock preparation following protocol in similar procedure in Chapter II section 2.2.1.

5.2.2 MOE1a cell line

MOE1a cell culture growth conditions following protocol in similar procedure in Chapter II section 2.2.2.

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**Figure 5.2** Probiotic direct and indirect benefit effect conveyed to oral cavity environment. The double end arrow representing the effect of the probiotic to oral biofilm and microflora and effect of both microflora and oral biofilm to the probiotics to function as probiotic (Courtesy of Meurman, J.H. (201). Probiotics: do they have a role in oral medicine and dentistry?. Eur J Oral Scie 113(3): 188-196.)
5.2.3 Cell morphology change

MOE1a cells \( (5 \times 10^5) \) were incubated with 200 moi probiotic microorganism and incubated for 24 hours at 5% CO\(_2\) and 37°C. A Zeiss Axio Vert A1 microscope was used to capture phase contrast images to determine any morphologic changes induced through the exposure to the bacteria.

5.2.4 MTT assay

MTT assay performed following protocol in similar procedure on Chapter II section 2.3.1.

5.2.5 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA assay performed according to manufacturer instruction and written in brief in Chapter II section 2.4.1.

5.2.6 Transepithelial electrical resistance (TEER) measurement

TEER assay was developed to determine epithelial integrity in vitro by measuring cell monolayer resistance to the passage of an electric current. Normal barrier function is characterized by increased or plateau electrical impedance and the opposite is applied to compromised epithelial layers. Cells were seeded in different concentration \( (10^3 \text{ to } 10^6) \) in 250 µl Defined Keratinocyte-SFM (Invitrogen 10744) medium in the upper chamber, and 600 µl in the lower compartment, and the resistance of the epithelial layer was measured every three days until it reached full confluence which was indicated by attaining the highest resistance that become plateaued, before decreasing due to overcrowding and cell death. The medium was changed every three days after each measurement.

Other experiment involving stimulation of fully confluent cells which started at a point where stable recordings reached. The fully confluent cells then exposed
separately to media only as control, heat in activated 20 moi \textit{E. coli}, 200 moi VSL#3 or combination of both. The experiment was performed in triplicate and repeated 3 times. TEER measurements were recorded in ohm (\( \Omega \)) after subtracting each read out with the background resistance of the membrane only.

TEER measurement technique was written in detail as per protocol on Chapter II section 2.6.

\textbf{5.2.7 F-actin fluorescence}

This assay aimed to observe F-actin dynamic reorganization which provides the driving force that direct cell migration. In culture environment, the oral epithelial sheets migrate and respond to some pathogens such as \textit{E. coli}. In this assay we also aimed to understand how the poly-probiotic VSL#3 modulates the F-actin structures in the MOE1a cells. In brief, \(10^3\)-\(10^5\) MOE1a cells were growth on glass cover slip, which were placed inside a 6 well plate until they reach semi confluent (60-70%). The media was then removed, and the cells gently washed with sterile PBS at RT. Formaldehyde 3.7\% (Sigma-Aldrich, UK) in PBS at RT was added to each coverslip for 10 minutes before another wash step. The cells then permeabilise using 200 µl of 0.01\% triton X buffer (Sigma, Cat no. T8787) in PBS for 5 minutes at RT. Wash step was repeated twice. F-actin was stained using phalloidin-ALEXA488 in combination with the nuclear stain 4’6-diamidino-2 phenylindole (DAPI, Sigma, Cat No.9542-10MG). 100 µl of 100 nM DAPI and phalloidin solution in PBS was incubated for 30 minutes at RT in the dark. The coverslips were then washed twice in PBS and twice in sterile water (each wash step included a 5-minute incubation with either PBS or water), the coverslips were removed, and mounted using antifade-PURE GOLD mounting media (Life Technologies, Cat no. p36941) on a glass slide and left
overnight at RT in the dark to dry. The slides were then viewed and imaged using a
Ziess Axio Vert A1 microscope.

Total fluorescence (488 nm) phalloidin was calculated using Fiji public domain
software (http://fiji.sc/). Fiji is an abbreviation for Fiji is just imageJ is a distribution
of ImageJ software that comes bundled with a range of add-ons intended primarily for
life science and which is contain all ImageJ software inside.(254) Total phalloidin
fluorescence was calculated by subtracting the value of mean fluorescence with
background value of the picture using formula:

CTCF - Corrected total cell fluorescence.

5.2.8 In vitro wound healing assay and time lapse imaging

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

Wound healing assay is a widely used assay to observe cell migration and
would closure.(255) Cell migration is a key property of live cells and critical for
maintenance in any epithelial disruption. Cell migration is not only important in normal
development, but also for complex immune responses, tissue repair and disease
process such as cancer metastasis and inflammation.(256)

In this study, three different technique were used to perform wound healing
assay. First, to observe cell motility and cell's morphological characteristics a
confluent monolayer of MOE1a cells were cultured in a dish containing a Ibidi insert
(Ibidi,Cat no. 80366). On removal of the insert cell migration was recorded by taking
phase contrast images at different time points using an inverted microscope with a 20
times magnification. The microscope does not need to be equipped with CO₂ supply
because the dish only put under the microscope for maximum of 10 minutes. This
technique proved to be quite challenging in term of taking images at the same exact
position of the created wound. Hence, this technique was used merely to observe the migration of individual cells.

The second wound assay technique employed used the Ibidi culture insert (3 columns per insert) to generating 500 µm defined cell-free gaps as an artificial open wound in a monolayer (figure 5.3A confluent monolayer of MOE1a cells were cultured in a dish containing a Ibidi insert (Ibidi,Cat no. 80366). On removal of the insert the confluent monolayer along with the gap was treated as written on 5.2.7 before visualising under an inverted microscope with a 10x magnification. Since the culture insert was only available in individual dishes, it was unpractical for multiple treatment comparison. Therefore, this experimental approach was used to emphasize the polybiotic effect on artificial wound healing closure.

The third technique, the scratch assay was performed as per protocol on Chapter II section 2.7. Briefly, MOE1a cells were cultured on a 6-well tissue cultured plate after it became confluent. A scratch was performed using a sterile 200 µl pipette tip (Figure 5.3A). The cells were then treated with 200 moi VSL#3, or 20 moi HkEc, or combination of both. Plate were then incubated in a Live imaging microscope equipped with a motorized stage, environmentally-controlled chamber 37°C with 5% CO2 supply), automated microscope with camera with multi-position image acquisition. After focus set up the scientific-grade digital camera with phase contract acquired images every 10 minutes for 48 hours with 20x magnification. Data analysis measured as written in Chapter 2 section 2.7.
Figure 5.3 Wound healing technique. A. Classic scratch assay using MOE1a monolayers. The scratch was performed using a sterile 200 µl tip pipette. B. Model wound assay implementing Ibidi culture insert 2 columns generating more define and uniform artificial wound.
5.3 Results

5.3.1 Poly-biotic VSL#3 has no toxic effect nor capability to trigger a robust immune response or a major morphological change in MOE1a cells

To determine whether the multi-species VSL#3 has a detrimental effect on oral epithelial cells, MTT viability assay was performed after stimulating the MOE1a cell line with a range of doses of the poly-biotic in presence or absence of the pathogenic bacteria HkEc. Live poly-biotic VSL#3 in the range of $1.6 \times 10^6$ to $10^8$ had no toxic effect on MOE1a cells, displayed by constant number of viable cells for all concentration after 24 hours of stimulation. The level of viable cells after VSL#3 exposure was similar to the levels observed for unstimulated cells (Figure 5.4).

The same effect demonstrated on MOE1a morphological changing. After 24 hours stimulation with 200 moi VSL#3 live or heat-inactivated alone, 20 moi HkEc or combination of both the cellular integration and morphology showed no differences (Figure 5.4)

![Figure 5.4](image-url)  
**Figure 5.4** Neither VSL# nor HkEc pathogenic bacteria affect MOE1a cells morphology. After reaching 90% confluent, oral epithelial layer stimulated with either VSL#3 200 moi or HkEc 20 moi. The representative images are shown from three independent experiments, magnification 10x.
5.3.2 VSL#3 has no toxic effect on MOE1a cell line model even with the highest concentration stimulation

As expected, incubation of MOE1a cells with a range of different concentration of poly-biotic VSL#3 has no significant effect on the cells viability (Figure 5.5A).

Moreover, exposure of MOE1a cells to a range of VSL#3 concentrations did not activate IL-6 cytokine secretion, save with the $10^9$ concentration. In contrast, 20 moi HkEc displayed extensive effect on immune activation as measured by IL-6 release. The induction effect could not be inhibited by incubation of VSL#3 first followed by HkEc stimulation even with the highest concentration. Interestingly, when stimulated in a different way where pathogenic HkEc first added into the cell monolayer following by $10^9$ VSL#3 concentration, the IL-6 secretion was fully inhibited. These suggesting activation of poly-biotic effect might be trigger by the pathogenic bacteria (Figure 5.5B)

Additionally, exposure with pathogenic HkEc for 24 hours strongly inhibited MOE1a proliferation compared to either unstimulated or VSL#3 alone-exposure. However, co-culture with poly-biotic VSL#3 could counteract inhibition effect to the level of unstimulated proliferation (Figure 5.6)
Figure 5.5 MOE1a cell viability and immune activation after 24 hours exposed to a range of VSL#3 doses plus or minus HkEc. A. Cell viability after stimulation with $1.6 \times 10^6$ to $10^8$ of live VSL#3 for 24 hours. B. IL-6 cytokine secretion resulting from the different doses of VSL#3 in the absence or presence of HkEc. Results expressed as mean ± SDev (n=8). Two-way ANOVA was used to performed multiple analysis with a result of ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. 
Figure 5.6 VSL#3 ameliorate cell viability after 24 hours stimulated with HkEc. Data performed once with eight biological replicates. Two-way ANOVA was used to performed multiple analysis with a result of ns: \( p > 0.05 \), \(*: p \leq 0.05\), \(**: p \leq 0.01\), \(***: p \leq 0.001\). Bars represent groups of treatment, dots represent number of wells.
5.3.3 MOE1a cells demonstrate a change in cellular area and f-actin levels after VSL#3 exposure.

Wound restitution is defined as a capacity of the epithelial cells for resealing superficial wounds and this process can be influenced by external (inflammation, infection) and internal (adhesion molecules and cytoskeletal regulation) factors.\textsuperscript{(257)} The restitution process requires cellular motility, which involves the reorganisation of the cytoskeleton and f-actin remodelling. We have shown that wound healing and cellular motility were altered in MOE1a cells exposed to VSL#3 plus or minus HkEc, therefore we investigated the potential alteration in the cells cytoskeleton \textsuperscript{(258)} using phalloidin a protein from the deadly mushroom Amanita phalloides, which binds specifically to f-actin structures in cells.\textsuperscript{(258)}

Our results revealed the presence of f-actin rich stress fibres were concentrated at the cells boundaries in untreated MOE1a cells (Figure 5.7.A). The stress fibres demonstrated no obvious alteration in cells exposed to the poly-biotic VSL#3 (Figure 5.7.B). In contrast, MOE-1a cells exposed to HkEc seemed to demonstrate a reduction in stress fibres and a loss and/or relocalisation of f-actin (Figure 5.7.C). After HkEc stimulation f-actin accumulates around the nuclei a structure that is not present in unstimulated or VSL#3 exposed cells. The combination of VSL#3 and HkEc seemed to result in the retention of the f-actin stress fibres and a lack of nuclei associated f-actin, suggesting that the poly-biotic inhibits the effect of the HkEc on the cytoskeleton (Figure 5.7.D).

A more quantitated measurement of change in f-actin was performed to determine the effects of bacterial exposure on MOE-1a cells. In term of the oral epithelial cell shape, we found that VSL#3 alone reduced cell area compared with unstimulated cells ($p < 0.05$) (Figure 5.7.E). The area was further reduced when the MOE-1a cells were exposed to HkEc (Figure 5.7.E). The co-incubation with VSL#3
and HkEc resulted in a recovery in cell area, demonstrating a potential inhibitory effect of VSL#3 on HkEc (Figure 5.7.E).

The alteration in cellular area could have a profound effect on the cytoskeleton and in order to assess this we calculated the total level of f-actin per cell. Our results demonstrated that unstimulated cells express the highest level of f-actin (total fluorescence intensity per cell of approximately 100,000 fluorescent units/µm²), suggesting a higher concentration of F-actin structures such as stress fibres. Exposure of MOE-1a cells to VSL#3 or HkEc resulted in a loss in fluorescence intensity (~45,000 and ~40,000 fluorescent units/µm² respectively) (Figure 5.7F). There was not significant difference between VSL#3 and HkEc treatment on f-actin levels. Interestingly, when the VSL#3 and HkEc were combined the total amount of f-actin significantly increased compared to the exposure to the single species treatment (~65,000 fluorescent units/µm²) (Figure 5.7.F).
Figure 5.7 F-actin organization on MOE1a cells. A. Stable, continuous, and collective F-actin organization on A. untreated cells. B. on VSL#3 with less collective area on the cells edge, C. disorganise F-actin on HkEc-treated cells, D. Re-stabilisation effect of VSL#3 after HkEc stimulation.
Figure 5.7 F-actin organization on MOE1a cells (continue). E. Change in cell surface area after poly-biotic VSL#3 stimulation, F. Alteration in F-actin levels per cell due to bacterial stimulation. Each symbol represents a single cell.
5.3.4 VSL#3 is a potent inhibitor of CXCL10, CXCL8 and IL-6

Epithelial cells are capable of mounting an immune response to bacterial and viral challenge as well as responding to immune stimuli within the tissue (e.g. IFN$_\gamma$ and TNF$_\alpha$). From the previous work presented in Chapter IV it was possible that VSL#3 could have an anti-inflammatory effect on the oral epithelial cells during immune stimulation. To test this MOE-1a cells were stimulated with either IFN-$_\gamma$ or HkEc in the presence or absence of VSL#3 and quantified the release of a range of pro-inflammatory cytokines (Figure 5.9). In all cases the MOE-1a cells did not release CXCL10, IL-6 or CXCL8 above the levels seen with unstimulated cells when exposed to VSL#3 for 24 hours. Whereas, exposure to IFN-$_\gamma$ or HkEc resulted in immune activation and the release of CXCL10 or IL-6/CXCL8 respectively. Co-incubating VSL#3 with IFN-$_\gamma$ or HkEc resulted in the loss in cytokine secretion. These findings demonstrate the anti-inflammatory capability of VSL#3.

5.3.5 VSL#3 maintains oral epithelial tight junction integrity and demonstrated competency to inhibit loosening of membrane integrity during HkEc exposure

Epithelial membrane integrity plays an important role in supporting the maintenance of a barrier at the mucosal surface. The epithelial layer is also uniquely positioned as a facilitator for crosstalk between mucosal immune cells and the complex external environment. Membrane integrity is maintained through a complex cell-to-cell interaction, which pathogenic bacteria are capable of disrupting. In order to test the potential effects of VSL#3 on oral epithelial integrity I performed trans epithelial electrical resistance (TEER) assays on MOE1a cells in the presence or absence of HkEc.
Figure 5.8 Cytokine secretion from MOE1a cells after 24 hours stimulation. A. CXCL10 level could not be detected with either HkEc nor LPS, but responded to 10 ng/ml IFN-γ, which was blocked by the co-incubation with VSL#3. B. IL-6 secretion after HkEc stimulation was blocked by the co-incubation with VSL#3. C. CXCL8 secretion after HkEc stimulation was partially blocked by the co-incubation with VSL#3. Experiments performed in three biological replicates, and data analyzed using two-way ANOVA multi-comparison with ns: p > 0.05, *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001.
Figure 5.9 Transepithelial electrical resistance measurement. A. The formation of a complete monolayer of MOE1a cells in the presence of different cells concentration B. Fully formed MOE1a monolayers with intact tight junction integrity were stimulated with VSL#3, HkEc and combination of both.
Figure 5.9A demonstrated that with a range MOE-1a cell concentrations (5 x 10^3 to 10^6) full confluency was reach by day 7 and maintained or slightly decreased by day 10. HkEc induced time-dependent reduction of TEER in a fully confluent monolayer (day 7) compared to either untreated or VSL#3-treated (Figure 5.9B). Interestingly, the HkEc reduction in TEER was counteract when VSL#3 was co-incubated. The loss in TEER demonstrates a loss in oral epithelial membrane integrity in the presence of HkEc. The fact that VSL#3 can overcome or block this demonstrates a potentially important role of probiotics in epithelial barrier maintenance.

5.3.6 The rate of closure of MOE1a monolayer cells co-cultured with VSL#3 could counter the slow closure caused by pathogenic HkEc

Oral mucosal healing is a crucial process for re-establishing epithelial barrier integrity following injury or infection. Wound healing is a complex process requiring a high degree of cellular orchestration. At mucosal surfaces the presence of microbes and microbial products will impact on the wound healing process. The activation of the immune system as well as direct toxicity will all influence the speed the epithelial barrier can be repaired. To determine if the presence of probiotics can influence the wound healing process a number of assays using the MOE-1a cell line were performed.

Fully confluent monolayers of MOE1a cells were grown in a dish containing the Ibidi insert or a scratch assay was performed with a sterile 200 µl pipette tip to create an artificial wound. The sterile wound was then exposed to 200 moi VSL#3 alone, 20 moi HkEc or combination of both.
Scratch assays demonstrated that the presence of VSL#3 had no significant impact on wound closure compared to unstimulated MOE1a cells (Figure 5.10). The inclusion of HkEc into the assay resulted in a delay in wound closure compared to both unstimulated and VSL#3 exposed MOE1a cells. Combining HkEc and VSL#3 overcame the inhibitory effect of HkEc and resulted in a wound closure similar to unstimulated cells. The inhibitory capacity of VSL#3 was further confirmed using the Ibidi insert assay (Figure 5.11 and 5.12). These results demonstrate that VSL#3 may have the ability to inhibit the effects of pro-inflammatory bacteria that are capable to slow down oral epithelial wound closure.

To determine if pro-inflammatory bacteria influenced the migratory speed of MOE1a cells during wound closure, individual cells were tracked using Image J software and an average speed per cell determined. Untreated cells reached a maximum migration speed of 24.7 µm/h (Figure 5.13A), whereas, cell-exposed to VSL#3 were found to be slower at 17.9 µm/h (Figure 5.13B). The inclusion of pathogenic HkEc had the most dramatic effect in migration rate resulting in a speed of 13.3 µm/h (Figure 5.13C). Interestingly, The co-incubation of VSL#3 with HkEc ameliorate the slow migration and reached resulted in a speed of 19.2 µm/h (Figure 5.13D). To normalize different width of wound closures, linear regression was run to compare the rate closure by slopes and intercept differences. The result demonstrated significant (P < 0.0001) slope dissimilarity between VSL#3 and HkEc, VSL#3_Ec and HkEc but had equal slopes for VSL#3 and VSL#3_Ec. These findings reinforce the inhibitory capability of VSL#3 against the effects of pro-inflammatory bacteria such as E. coli.
Figure 5.10 Classic scratch assay with phase images at different time points. A. Representative figure of wound bed closure of MOE1a monolayer after exposed with VSL#3 200 moi, HkEc 20 moi, or combination of both. B. Either in the absent of any bacteria or with VSL#3 added, the MOE1a cells demonstrated a rapid wound closure by 48 hours. Wound closure was not reached at 48 hours in the presence of pathogenic bacteria HkEc. The HkEc effect was nullified by co-incubation with VSL#3 with total wound bed closure taking 48 hours. There was no statistical significant difference on the speed of wound closure between unstimulated, VSL#3 and HkE treated on combination of both when calculated using two-way ANOVA. This scratch assay repeated in triplet technical experiments with two biological replicate each and total six snapshot picture included in analyse for each experiment.
Figure 5.11 Ibidi insert with snapshot pictures. A. Cells migration of MOE1a cells displaying cells restitution with more collective monolayer sheet on VSL#3 treated cells compared to HkEc which migrated more individually. B. Graph bar of migrated cells between two groups. The experiment performed once with two wound bed replicates origin from one Ibidi column and six different point snapshots.
Figure 5.12 Ibidi column insert with phalloidin stain. In this wound healing assay, Alexa 488-phalloidin was used to label the abundant F-actin in the cytoplasm and used to determine the speed of the wound closure after the monolayer was untreated or treated with either VSL#3, HkEc, or combination of both. ImageJ software then applied to emphasise wound bed closure visually. Monolayers exposed to pathogenic HkEc demonstrate a delayed wound closure, however this effect could inhibited with the co-culture of VSL#3.
Figure 5.13 MOE1a migration rate with different stimulants. A. Untreated cells, B. VSL#3 exposed, C. Pathogenic HkEc. D. Co-culture VSL#3 and HkEc. To normalize different width of wound closures, linear regression was run to compare the rate closure by slopes and intercept differences. The result demonstrated significant slope dissimilarity with P < 0.0001 between VSL#3 and HkEc, VSL#3_Ec and HkEc but has equal slopes for VSL#3 and VSL#3_Ec. Rate of sheet migration from all treated monolayer cells are significantly different from the unstimulated one.
5.4 Discussion

To understand more about probiotics as a functional food, i.e. food with nutrition value with an added health benefit, we carried out several assays addressing issues regarding probiotic mechanism of action on oral epithelial cells. The first issue regarded safety of the probiotic regarding the toxicity on the oral epithelial cell line. Even at the highest concentration of $10^8$ bacteria no toxic effect could be identified both in regard to epithelial cell viability alteration in tight junction integrity. These findings are relevant to VSL#3 as the current commercially available sachets contain very high doses of bacteria ($10^{14}$ per sachet) which means that the oral and gastrointestinal epithelium could be exposed to high levels. Theoretically, probiotics could be adaptable to oral conditions judging from the fact that some well-known probiotic species have an oral origin such as *Streptococcus thermophilus*. Having screened two independent studies for beneficial microorganism in oral environment, we demonstrated that the oral environment is a normal habitat for some known probiotic species. We were able to identify up to 8 species (0.8%) of probiotic bacteria in the mouth of healthy subjects (Chapter IV Figure 5). We were therefore interested in how probiotics may influence the immune response and wound closure of the oral epithelium.

Pro-inflammatory cytokines are thought to be a critical driving factor in several oral/mucosal diseases, such as OLP. OLP is characterised by lymphocytic infiltration along with the presence of macrophages in the basal lamina of the oral epithelium. There is evidence of an associated elevation in pro-inflammatory cytokines such as IFN-γ, TNF-α, CXCL8, IL-6 and CXCL10. (7, 180) Our previous OLP screening study provided support for an elevation in
pro-inflammatory cytokines in both the circulation and saliva in patients with OLP (Chapter III, section 3.3.4. It is possible that oral epithelial cells contribute locally by secreting pro-inflammatory cytokines and this could influence disease severity. Exposing the oral epithelium to probiotic bacteria may facilitate a reduction in the proinflammatory cytokine levels and aid in the healing of oral inflammation and ulceration. Our in vitro results demonstrated that MOE1a cells were unable to secrete CXCL10 when stimulated with HkEc, whereas exposure to IFN-γ strongly stimulated the release of this protein. The elevation of CXCL10 is strongly associated with OLP\(^{(25, 158, 169, 171)}\) and if the oral epithelium contributes to the raised levels identified in the saliva and circulation, it probably does not involve direct stimulation by bacteria, but through an alternative stimulus such as IFN-γ. Activated effector T cells which are known to be elevated in OLP are a potential source of raised IFN-γ in the oral mucosa. The potential inhibition of CXCL10 chemokine displayed with co-culturing IFN-γ and VSL#3 suggests that this may be clinically beneficial to patients with OLP. The inhibitory capacity is unlikely to be due to the blockade of TLR or NLR on the MOE-1a cells as IFN-γ immune activation will occur through the IFN-γ Receptors (IFNGR1/2). It is therefore likely that VSL#3 induces an inhibitory signal that interferes with the pro-inflammatory signalling within the MOE-1a cells. This pathway and relevant receptors are still unknown. In addition to CXCL10, a similar inhibition in the secretion of IL-6 and CXCL8 was also evident when MOE1a cells were stimulated with HkEc in the presence of VSL#3. These results were consistent with a number of previous reports of VSL#3 effects in vitro and in vivo.\(^{(111, 213, 260, 261)}\) However, Singh et al, reported that there was no significant change in plasma pro and anti-inflammatory cytokines of IFN-γ, IL-
4, IL-5, IL-10 and TNF-α before and after 4 weeks of probiotic VSL#3 consumption. However, they did reported that the cytokine levels in supernatants from LPS-stimulated whole-blood cultures showed a significant reduction in IL-10, IL-6 and TNF-α.\(^{(262)}\) It should be noted that the study by Singh et al investigated the effects of VSL#3 on healthy women who did not have a raised cytokine profile due to chronic inflammatory disease.

Another important issue regarding mechanism of action of probiotics in oral environment is the regulation of mucosal permeability. Mucosal permeability conveys a number of important functions such as, filtering and transporting molecules and ions, solutes and water from and to the underlying layers. Mucosal permeability is partially regulated by multi-protein complexes defined as tight junctions (TJs).\(^{(263)}\) TJs also regulate a variety of signalling and trafficking molecules, regulate cell differentiation, proliferation and polarity. TJs are comprised of protein complexes that contain occludin,\(^{(264)}\) occludin 1B,\(^{(265)}\) zonula occluding protein 1 and zonula occluding protein 2,\(^{(266)}\) E-cadherin, as well as members of the protein family of claudins.\(^{(264)}\) Oral mucosal tissue from OLP patients have previously been shown to expressed significantly reduced levels of claudin-1, claudin-4 and E-cadherin compared to healthy control.\(^{(267)}\) The change in TJs architecture can have a major influence on epithelial permeability and could result from interactions with pathogenic bacteria namely *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*.\(^{(268)}\)

TEER is a measure of mucosal barrier permeability *in vitro* which can be used to determine changes in TJs and cell-cell contacts. Our results demonstrated that VSL#3 has minimal effect on membrane integrity of the oral epithelial cells and can even counteract the membrane destabilizing effect of
the pathogenic *E.coli* on MOE1a monolayers. Our findings agree with a previous report on a range of different probiotic strains on regulating tight junction proteins. La Fata, listed some of the single species with the capability to induce or maintain TJs-associated protein. Single species which they identified were *Escherichia coli* Nissle 1917, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* DN_114001, *Streptococcus thermophiles* ATCC 19258 and *Lactobacillus acidophilus* ATCC3456, *Bifidobacterium infantis* (From VSL#3 cocktail), *Lactobacillus plantarum* MB452, *Lactobacillus plantarum* WCFS1, *Lactobacillus plantarum* CGMCC No. 1258.

Furthermore, our study demonstrated that other cytoskeletal architectural changes were evident when MOE1a cells were exposed to VSL#3. The level and distribution of f-actin was affected by the probiotic, which suggests that epithelial cells are responding to the presence of the probiotic and altering their cytoskeleton but maintaining membrane integrity. F-actin is the most abundant protein in most eukaryotic cells. Its function, along with the other actin binding proteins, are involve in cell motility and the maintenance of cell shape and polarity.

Our *in vitro* study clearly showed that the effects on oral epithelial cells derived from membrane protein of the VSL#3 species since we stimulated the cells using heat killed probiotic species. *In vivo* the effects of VSL#3 on epithelial barrier may be different and several reports have identified some consequences of probiotic consumption. DeAngelis, 2005 reported that the poly-biotic VSL#3 increased the activation of zonulin, leading to cytoskeleton reorganisation and intestinal permeability with respect to non-hydrolysed wheat gliadins. Wheat gliadins activate target receptors in the intestinal wall ws in a manner similar to
the zonula occludin toxin (ZOT toxin) produced by cholera. Increased concentration of serum zonulin is a sensitive indicator of the increased permeability of the intestinal wall, and probably oral epithelia, and is increased in several autoimmune diseases, including celiac disease, type 1 diabetes, non-alcoholic liver disease, rheumatoid arthritis and multiple sclerosis.\textsuperscript{(271, 272)} The underlying mechanism of VSL\#3 capacity to degrade gliadin polypeptides which was consider as the trigger for celiac sprue is the enzyme specific for prorich peptides and general aminopeptides which were largely distributed in the probiotic VSL\#3 preparation. The capability of this multi-biotic to hydrolyse gliadin was lost when the individual strains of VSL\#3 were tested. These results suggest a synergistic mechanism of action of VSL\#3.

Our wound healing study demonstrated a more stable f-actin cytoskeleton in MOE1a cells after VSL\#3-treatment versus HkEc cells which suggests a potential difference in cellular motility. The wound healing rate was determined by three complementary techniques which employed different ways to observe the cells migration. All techniques, however, demonstrated the potential of VSL\#3 to ameliorate the rate of wound healing in the presence of the pro-inflammatory HkEc. Wound healing assays measure the collective cell migration known as sheet migration.\textsuperscript{(273)} The sheet migration involves a complex interplay among mechanic force, molecular interactions and biochemical cascades that are triggered by stimulation of the cell monolayer. These assays reveal the rate of gap closure, and cell migration numbers and morphology. The MOE1a cells morphology does not change even in present of pathogenic bacteria HkEc. However, HkEc-treated MOE1a cells movement was more individual compared to VSL\#3-treated. The individual movement suggests
weaker TJs between the cells that could account for the identified increase in epithelial permeability, reduced membrane integrity and slower wound healing. Interestingly, the co-culture of VSL#3 with pathogenic HkEc reverses all of these epithelial phenomena and provides support for the possible use of the poly-biotic VSL#3 as a treatment in diseases like OLP.
CHAPTER 6
EFFECT MULTI-BIOTIC VSL#3 ON ORAL LICHEN PLANUS: A DOUBLE-BLIND RANDOMIZED PLACEBO-CONTROLLED FEASIBILITY TRIAL

6.1 Introduction

OLP is an inflammatory disease of the mucosal tissue and skin. Currently the aetiology is unknown.\textsuperscript{(4, 14)} Some authors suggest an abnormal immune response resulting from a defect in a group of proteins named Toll Like Receptors (TLRs).\textsuperscript{(45, 108)} The main function of TLRs is the recognition of microbial derived molecules, which upon engagement results in the activation of the immune system and localised inflammation. A major site of TLR activity occurs at mucosal surfaces including the oral mucosal. TLR activation results in the upregulation and secretion of proteins called chemokines and cytokines that recruit leukocytes to the mucosal tissue.\textsuperscript{(274)} Under certain circumstance this response can become deregulated leading to an elevated response which has the potential to drive disease pathologies, such as OLP.\textsuperscript{(31)}

There remains no curative therapy for OLP and therefore management is aimed at reducing painful symptoms, which is typically achieved through healing of mucosal erosion and ulceration. Overall, both topical and systemic therapy of OLP is burdened by adverse side effects including immune suppression, which is associated with an increased risk of infections and cancer, and hepatotoxicity. More importantly, although a symptomatic improvement is often observed, there remains little robust evidence supporting the use of current therapeutic modalities due to the lack of high-quality well-designed clinical trials.\textsuperscript{(11)} Finally, in patient’s point of view, the effectiveness of
therapy is limited and worsened by the frequent re-activation/relapses of the disease.

A probiotic is defined as a live microorganism that confers a health benefit on the host. These agents have historically been used to treat diarrhoea and extensive research has been conducted to provide the evidence to support the benefit of probiotics to human health. PubMed has indexes more than 25,000 research articles studying probiotics. There is support as a potential viable alternative for therapy either as a single dose or as adjunctive in regulating inflammatory cytokines in some diseases. Table 6.1 demonstrated the eight species contain in VSL#3. VSL#3 is marketed in the UK as a food supplement and has been listed in the British National Formulary (BNF) up to 2019 first year editions with indication for the maintenance of remission of ileoanal pouchitis in adults. It is also available over the counter and distributed by Ferring Pharmaceutical Ltd.

**Table 6.1 VSL#3 composition in one sachet**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Code</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Streptococcus thermophilus</td>
<td>BT01</td>
<td>Yogurt/dairy origin</td>
</tr>
<tr>
<td>2 Lactobacillus paracasei</td>
<td>BP07</td>
<td>Cheese/diary origin</td>
</tr>
<tr>
<td>3 Lactobacillus delbrueckii subspecies bulgaricus*</td>
<td>BD08</td>
<td>Cheese/diary origin</td>
</tr>
<tr>
<td>4 Lactobacillus acidophilus</td>
<td>BA05</td>
<td>Healthy adult human</td>
</tr>
<tr>
<td>5 Lactobacillus plantarum</td>
<td>BP06</td>
<td>Fermented plants</td>
</tr>
<tr>
<td>6 Bifidobacterium breve**</td>
<td>BB02</td>
<td>Healthy infants</td>
</tr>
<tr>
<td>7 Bifidobacterium longum**</td>
<td>BL03</td>
<td>Healthy infants</td>
</tr>
<tr>
<td>8 Bifidobacterium infantis</td>
<td>BI04</td>
<td>Healthy infants</td>
</tr>
</tbody>
</table>

* Now classified as Lactobacillus helveticus
** Now classified as Bifidobacterium animalis subspecies lactis

Supplemental material: maltose, cornstarch, anti cracking agent: silicon dioxide
There is currently a dispute between two rival companies regarding the VSL#3 product and an alternative brand called Vivomixx. The dispute concerns the manufacturing and composition of the VSL#3 produced after 2016, which is claimed to be different from the pre-2016 product that was used for studies and clinical trials published. This issue is extensively covered in appendix 3 and includes some comparative studies between VSL#3 and Vivomixx. In all of the studies reported in this thesis the post-2016 VSL#3 was used and is referred to as VSL#3.

In a mouse model of colitis, VSL#3 could alleviate dextran sulphate sodium (DSS) - induced colitis by regulating T follicular helper (Tfh) cells. The regulating Tfh mechanism achieved via increased Bc16 mRNA and decreased Blimp-1 mRNA level of mesenteric lymph nodus (MLN) and colon of the mouse model. Bc16 is the master transcription factor that regulates the differentiation of Tfh cells, while the Blimp-1 is an antagonist. This could indicate how VSL#3 reduces symptoms in human UC. Moreover, in a double-blind, placebo-controlled trial performed in intensive care unit of a tertiary care teaching hospital, North India, using the pre-2016 VSL#3 from VSL Pharmaceutical, Towson, MD resulted in a significant decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines in children with severe sepsis. VSL#3 has also been shown to have the ability to maintain or improve membrane barrier function, and modulate the gut microbiota composition in type 1 diabetes mouse model.

A potential mechanism of action of VSL#3 on cytokine secretion was described by Hormansprenger et al and involved the blockade of intracellular cytokine trafficking. They reported that VSL#3 could directly inhibit CXCL10.
secretion, a cytokine which in our previous screening study (Chapter 3) – was over expressed in patients with OLP. Taken together, in vitro and in vivo research suggested that VSL#3 administration was effective,\(^{(214)}\) changing microbiota composition,\(^{(189)}\) modulating immune cells,\(^{(279)}\) and maintaining / improving epithelial barrier function.\(^{(221)}\)

In summary, studies suggest that the probiotic VSL#3 is a potent regulator of inflammation capable of changing or controlling the inflammatory cytokines within the mucosal layer. We also know that the oral mucosa conditions can be influenced by probiotics as a preliminary report that used *Lactobacillus brevis CD2* lozenges in Bechet’s disease, combination of *Lactobacillus acidophilus* and *lactobacillus bulgaricus* for recurrent aphthous disease (RAS) and combined poly-biotic of *Bifidobacterium longum*, *Lactobacillus lactis*, and *Enterococcus faecium* for oral mucositis resulted in reducing the number of oral ulcerations and subjective relief of oral discomfort, and the severity or oral mucositis.\(^{(123, 125, 280)}\) To date there have been no attempts to investigate the potential of probiotics in the treatment of OLP, even though there are strong similarities between intestinal gut and oral mucosa. During our study a review was published which put forward an identical theory to ours but to date no clinical data has been published.\(^{(218)}\) There have been two OLP studies that have used probiotics and these will be discussed in relation to our findings in the conclusion.\(^{33, 104}\)

The oral mucosal layer is the first part of the gastrointestinal tract and provides a protective barrier between the environment and the host. In addition to the physical barrier the oral mucosa also has an immune function, which is conserved throughout the entire gastrointestinal tract and other mucosal
surfaces such as the lung. The epithelial cells contain TLRs and secrete anti-microbial peptides.\(^{(31)}\) In addition, the mucosal layer contains mucosal association of lymphoid tissue (MALT) that are diffuse concentrations of lymphoid cells (T cells, B cells, and macrophages), which are capable of mounting an immune response upon microbial challenge.

Based on current data on the clinical benefits of VSL#3 and probiotic in general, in a range of mucosal inflammatory diseases (Ulcerative Colitis, Bechet’s and RAS), together with the published evidences of its modulating effects upon the expression of inflammatory cytokines, and from our in vitro results on both THP-1 and MOE1a cell lines we have hypothesised that VSL#3 might have potential to modulate inflammation in OLP, which would translated into clinical benefits for participants.

6.2 Research plan

6.2.1 Objectives

6.2.1.1 Main objective

This study title was The Clinical And Biological effects of the use of pRobIOtic VSL#3 in patients with oral lichen planus: a proof-of-concept, acronym - CABRIO. The main objective was to determine whether the 30 days of VSL#3 probiotic supplementation has an effect upon painful symptoms of oral lichen planus measured by pain numeric rating score (pNRS).

6.2.1.2 Secondary objectives

The secondary objectives measured the efficacy of VSL#3 as an adjunct to other topical treatment on:
1. Disease activity which measured according to Guy hospital's oral disease severity score (ODSS)
2. Quality of life (QoL) of participants following the Chronic Oral Mucosal Diseases Questionnaire (COMDQ) score.
3. Safety and tolerability to the participants toward the high concentration of probiotic by observing the adverse events
4. Participant’s acceptance by examine compliance with therapy and attrition.
5. Association with changes in the serum and saliva proinflammatory cytokines
6. Associated metagenomics oral changes.

6.2.2 Outcomes

6.2.2.1 Primary outcome

The primary outcome was the change in pain score from baseline to the 30 days of poly-biotic consumptions.

6.2.2.2 Secondary outcomes

1. Change in severity disease from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.
2. Change in Quality of life from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.
3. Safety and tolerability from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.
4. Participant’s acceptance through compliance with therapy and attrition from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.

5. Associated changes of serum and saliva proinflammatory cytokines from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.

6. Associated metagenomics changes from each time point of 30 of VSL#3 consumption and day 60 / day 30 without poly-biotic consumption.

6.2.3 Research methodology

6.2.3.1 Ethic approval

This study has been approved by NHS Health Research Authority with lead sponsor University College of London under London-Queen Square Research Ethics Committee (REC). The REC reference number was 17/LO/0475, protocol number 16/0622, and IRAS project ID 22017. This study was also registered on ClinicalTrials.gov with identifier number (NCT number): NCT03052179.

Internally, this study was covered under the award number 174235, and UCL data Protection Registration of Z6364106/2017/02/21 clinical research.

6.2.3.2 Study design

The study design was a randomized, double-blind, placebo-controlled clinical trial with 30 days intervention to assess the effect of the multi-strain probiotic VSL#3 on oral lichen planus sign and symptoms.
6.2.3.3 Investigational product and funding sources

This study was funded by Ferring pharmaceutical Ltd Company, who marketed VSL#3 in the UK. The funding was used for participant award, travel reimbursement, and associated laboratory costs. Ferring also provided investigational product (VSL#3 and placebo), which were in plain-sachets and labelled based on randomisation number. The packages had expiry dates of April 2019.

The investigational study product contained placebo and active multi-strain probiotic. The multi-strain probiotic was a commercial brand named VSL#3 that contained eight different strain with high-concentration number of 450 billion per sachets (4.4 g). The strains listed were Lactobacillus acidophilus (BA05), Lactobacillus delbrueckii subsp. Bulgaricus (BD08) (reclassified as lactobacillus helveticus product label information), Lactobacillus paracasei (BP07), Lactobacillus plantarum (BP06), Bifidobacterium longum (BL03), Bifidobacterium infantis (BI04) (BL03 and BI04 reclassified as B. animalis subsp.lactis product label information), Bifidobacterium breve (BB02), and Streptococcus thermophilus (BT01). They also included maltose, cornstarch, and anti-cracking agent silicon dioxide. Placebo just contained maltose, cornstarch, and anti-cracking agent silicon dioxide.

OLP patients were prescribed four sachets a day to be divided equally and consumed in the morning and evening. The dosage based on maximum manufacturing recommendation. Duration of consumption of 30 days was decided upon based on the length of time that the study medication was expected to have a detectable effect as per manufacturer recommendation.
a quality control, a certificate of analysis and certificate of conformity were
provided by the manufacturer.

6.2.3.4 Study participants

Potential participants identified through the Oral Medicine out patients
clinics at the University College of London Hospital-Eastman Dental Hospital by
the Principal Investigator, co-investigators, and clinical care team. Relevant
data regarding past medical history, diagnostic tests and investigations
extracted with participant's consent from their clinical records by principal
investigator or co-investigators, who are part of clinical care team or have
honorary UCLH/NHS contract and GCP training.

6.2.3.5 Study inclusion and exclusion criteria

Inclusion criteria

1. Biopsy-proven diagnosis of OLP as per WHO histological criteria \(^{(281)}\) with
   no evidence of oral epithelial dysplasia or malignancy.
2. Presence of painful oral symptoms associated to OLP, with minimum
   severity of pain being ≥ 3 on a 0-10 numeric pain rating scale at screening
   and confirmed at recruitment/start of the intervention.
3. Age >18 years and willing to participate into the study.
4. Receiving no therapy or receiving best standard topical therapy (typically
   topical corticosteroids or immunosuppressant) with the exclusion of
   systemic corticosteroids or systemic immunosuppressant

Exclusion criteria

1. The use of systemic antibiotics, retinoid, corticosteroids or
   immunosuppressant within four weeks prior to enrolment in the study.
2. Pregnancy* or receiving IVF treatment.

3. Individuals with known history of systemic disorders affecting the immune system (e.g., connective tissue disorders or lupus).

4. Active cancer or cancer in remission undergoing maintenance with chemotherapy or immunomodulatory agents.

5. Evidence of oral epithelial dysplasia or oral malignancy on previous biopsy

* Evidence of negative pregnancy test at screening/randomisation visit (strip urine test) in women of child-bearing age in which the possibility of being pregnant cannot be otherwise excluded.

**6.2.3.6 Sample size calculation**

As a feasibility study the sample size of 30 participants has been chosen pragmatically. 30 participants is realistically achievable within the timeframe of the study and the capacity of the Oral Medicine clinics at UCLH EDH and is considered adequate for such early stage pilot work.\(^{(282)}\)

**6.2.3.7 Study recruitment**

The study procedures began with Identification of potentially eligible participants where potential participants identified in routine Oral Medicine clinics and provided with verbal and written information regarding the study. They were given adequate time to decide whether to participate (>24 hours). If the participant agreed to participate, they would be assessed against entry criteria, and if eligibility is confirmed, would be consented and recruited. Participants will then be allocated to one of the two study of therapy arms and given a study number (randomization).
30 individuals who consented were randomised into two groups: group A (15 participants) receives the VSL#3 and group B (15 participants) receives a placebo. A centralised computer-generated randomization list provided by an independent third party was used to conceal allocation of participants to the treatments. The list was sent to the drug manufacturer who labelled the VSL#3 or placebo with relevant study participant code, which came in the same identical plain-packages to ensure blinding (neither the participants nor the investigator were aware of the sachets content). The study statistician was also blinded to the allocation. Participants were allowed to use best standard therapy during the study.

6.2.3.8 Recruitment rate

Based on clinical observation in 2014, there was about 1200 appointments for OLP in the year. Ulcerative condition which was a requirement for this study was calculated based on patients record and epidemiology publications to be about 20 percent or 20 participants per month.\(^{(283, 284)}\) I recruited an average of 2 participants, fully eligible for this study, every month, and completed the recruitment in 12 months which was substantially faster than the supposed 15 months study period.

6.2.3.9 Randomisation procedures

A centralised computer-generated randomization list provided by an independent third-party statistician was used to conceal allocation of participants to the treatments. The list was sent to the drug manufacturer who labelled the VSL#3 or placebo packages with the relevant study participant code. All other aspects of the packaging were identical to ensure blinding
(neither the participants nor the investigator were aware of the package content). The randomization list was blocked using varying block sizes and 1:1 allocation to the treatment and control groups. The study statistician was also blinded to the allocation following participant consent, and confirmation of eligibility, the registration/randomisation procedure described was carried out.

The third-party statistician also sent the randomisation key to the person in charge of study site. This key was available 7 (seven) days a week in case of an emergency. The study site received the randomisation key in sealed envelopes one for each of the participants, containing information on which group they had been assigned (VSL#3 or placebo). These envelopes were held in a locked location by Professor Stephen Porter (Director of the Eastman Dental Institute) and were to only be opened in an emergency (un-blinding).

6.2.3.10 Measurement of response variables

6.2.3.10.1 Pain numeric rating scale (pNRS)

pNRS is a numerical score on a 10 cm horizontal line with the number on a scale from zero which represent no symptoms to ten representing the worse imaginable symptoms. The pain score was an average of the pain score in the last two weeks. This scale has been previously validated for measurement of the intensity of symptoms and indicator for pain in OLP.\(^{66, 165, 285}\) For every visit, participants were asked to mark the number on the pain scale according to their conditions in the last two weeks.

6.2.3.10.2 Oral disease severity score (ODSS)

ODSS was used to measure clinical signs of OLP which was a modification of the Escudier scoring system for oral mucosal disease developed
by Guy’s and St Thomas hospital of Kings College London Dental Institute. On oral examination, the predominant clinical type was recorded and scaled based on the site and activity please see figure 3.3 in Chapter 3.

6.2.3.10.3 Quality of life

The World Health Organization (WHO) has defined quality of life (QoL) as an individual perception of their position in life in the context of the culture and value system in which they lives and in relation to their goals, expectations and standards and concerns. We used Chronic oral mucosal disease questionnaire (COMDQ) which was composed of 26 oral-medicine specific QoL questioners developed in an Irish populations. The 26 questions are classified into 4 domains i.e. pain and functional limitation, medications and side effects, social and emotional, and patients support. The response scale ranged from ‘not at all’ to ‘extremely’ with accumulative scores from 0 to 100 where 0 as the highest quality index participants can go through and 100 as the lowest quality of life index. This QoL instrument has been validated and considered as reliable for patient-reported outcome measure for chronic oral mucosal disease in the UK population.

6.2.3.10.4 Safety and tolerability

Safety and tolerability of the participants toward the study material either a high concentration of probiotic or placebo was determined by recording associated and non-associated adverse side effects listed by the participants and asked on detailed anamnesis during each visit.
6.2.3.10.5 Acceptance

Participant’s acceptance to the high concentration of probiotic observed through compliance with therapy and participant attrition from the study.

6.2.3.10.6 Adverse event

Adverse event is any untoward medical occurrence in participants that does not necessarily have a causal relationship with the intervention method. We divided these into two categories i.e., expected (associated) or unexpected (non-associated).

Bloating is the expected adverse events which are side effects which would be consistent with the information listed by VSL#3 manufacturer on their website (https://www.vsl3.co.uk/order-now/faqs-main). The unexpected events were classified as side effects that might occur but had no rational association with the investigational product such as a car accident.

Details regarding adverse events, definition, category, recording and reporting of serious adverse events are listed on the ethic protocol approved by REC.

6.2.3.10.7 Circulating cytokine

Circulating cytokine levels measured by peripheral blood serum as per protocol on Chapter 2, section 2.5.3 at baseline, 30- and 60-days using ELISA and MSD.

6.2.3.10.8 Saliva cytokine levels

In this study, we measured saliva cytokine levels as a representation of the local immune response within the oral cavity and as a readout of the
changes induced by the consumption of the probiotic. Cytokine measurements were taken from the saliva samples collected as per protocol on Chapter 2, section 2.5.3 at baseline, 30- and 60-days using ELISA and MSD.

6.2.3.10.9 Changes to the saliva microbiome

Dysbiosis of the oral microbiome has been implicated as one contributing factor in the severity of OLP lesion. As the technology of high-throughput sequencing-dependent metagenomics became advance, we interested to identified whether there is any difference in metagenomics environment after 30 days use of high-concentration of probiotic.

6.2.3.11 Sample processing and analysis

In the study, blood and saliva collected from participants in accordance with the participant consent form and participant information sheet. After labelling, saliva sample transported to UCL Rayne Institute, molecular medicine laboratory within 5 hours and stored in a -20°C freezer. Samples thawed at a later date and the microbial DNA extracted. Once extracted the microbial DNA stored in a -20°C freezer in UCL Rayne Institute. Extract DNA purified by a conventional bacterial DNA extraction kit (Puregene DNA isolation Kit, Qiagen). The DNA subjected to PCR with primers designed to anneal to the universally conserved regions V5-V7 of the ubiquitous 16S rRNA gene Forward 5′CGGTTACCTTGTTACGACTT3′ and Reverse 5′AGAGTTTGATCMTGGCTCAG3’ primers. This gene acts as a phylogenetic marker for known bacterial species. The samples sequenced at UCL Genomics using their Illumina MiSeq next generation sequencing platform. Following
sequencing data then de-multiplexed, and the data for the individual subjects compiled and analysed.

The blood samples transported to the UCL Rayne Institute, molecular medicine laboratory, SLMS Faculty within 5 hours of collection and immediately centrifuged at 2000 rpm for 30 minutes; the serum removed and stored at -80°C until needed. The remaining blood pellet was disposed of following the standard procedures for handling hazardous biological material. Combination of multiplex and single cytokine assays were used (Meso Scale diagnostics pro-inflammatory cytokine assays and specific ELISAs from R&D Systems) to determine the serum concentrations of IL-6, IL-8, CXCL10, IL-1β, and IFN-γ. The assay followed the protocols according to manufacturer recommendations (Chapter 2. Sections 2.4.1 and 2.4.2).

6.2.3.12 Discontinuation/withdrawal of participants

Participant could have been withdrawn from the trial at any time, but the reasons for doing had to be recorded. Reasons for discontinuing the trial could have included:

- Disease progression whilst on therapy
- Intercurrent illness
- Participants withdrawing consent
- Persistent non-compliance to protocol requirements
- Feeling nausea, or vomiting related with the probiotic consumption
- Antibiotic or systemic corticosteroid or immunosuppressant during the clinical trial.
The decision to withdraw a participant from treatment was recorded in the CRF and medical notes. If a participant explicitly states they did not wish to contribute further data to the trial their decision must be respected and recorded in the CRF and medical notes.

6.2.3.13 Statistical Considerations

6.2.3.13.1 Primary Outcome

The primary outcome was the numeric pain score (NRS) measured at 30 days from treatment on a scale of 0 to 10 (worst possible pain). I analysed the 30-day pain score as a continuous score.

6.2.3.13.2 Secondary outcome(s)

Secondary outcomes are disease activity score (ODSS) and COMDQ at 30- and 60-days post treatment. These were analysed as a continuous score. The ODSS basically divides the oral cavity into 17 sites, and criterion-based numerical scores for each site given. The variables used were extent of the lesion (site score) and severity of the lesion at each site (activity score). Using this methodology, the maximum score possible for a site was 24, activity was 72. For mechanistic outcomes, changes in saliva and serum cytokines were calculated before and after VSL#3 consumption and analysed using linear regression adjusted for baseline.

6.2.3.13.3 Statistical analysis

Statistical analysis was conducted under the supervision of the clinical trials statistician. The information below provides a summary of the main analyses that was carried out,
Participant baseline data including demographics and clinical characteristics summarised by randomised group using means (with standard deviation), medians (with interquartile range) or frequency (with percentage) as appropriate. A Consort diagram described the flow of participants through the trial including rates of attrition.

Figure 6.1 Flow chart for CABRIO study design
The 30-day pNRS between randomised groups compared using analysis of covariance to adjust for baseline. The treatment effect estimate adjusted the difference in means which reported with a 95% confidence interval (95% CI) and P-value. Assumptions would be investigated and if these are not met a suitable alternative method would be identified. Analysis of the 60-day pain scores took a similar approach. Descriptive statistics and graphs used to examine the changes in mean pain score over time in each randomised group (including scores at baseline, 15-day, 30-day and 60-day follow up). Analysis performed by intention to treat.

The OLP disease activity score (ODSS) and COMDO analysed using the same methods as described above for the pain rating scale. To assess safety, occurrences of adverse events summarised by randomised group, using counts and proportions. We will consider whether the intervention is acceptable by summarising the compliance information obtained from participant diaries. Compliance was measured by participant’s diary and sachets left for each of the two follow up on day 15 and day 13.

In general, doing analysis on randomized controlled trial (RCT) data transparencies should on step by step and logical scientific background on the analysis. It is not easy to translate statistical result to the meaning of the clinical and biological process represented by the mathematical model used in statistic formulation. Statistical method used would depend largely on what research questions that need to answer, study design and sampling technique applied, and mainly type of the collected data.

In this analysis comparison of primary and secondary outcomes between the randomised groups was carried out using regression models as
these allow adjustment for the baseline measure of the outcome which is known to provide a more efficient analysis. A two sample t test was used for unadjusted comparisons of continuous variables between groups for normally distributed data and Mann-Whitney U test was used for skewed data. P values less than 0.1 were considered significant.

In representing the results descriptive statistics are used to describe the characteristics of the study sample and graphs are used to illustrate changes in our outcome measures over time in the placebo and active groups. Graphs of paired data also demonstrate the pre-and post-intake of the active/placebo management.

6.3 Results

6.3.1 Recruitment period

The participant recruitment finished earlier than we expected. From 15 months to 12 months as showed on figure 6.2.

6.3.2 Baseline

CDR (Clinical Data Repository) screening was applied to identify potential participants with OLP who were outpatients at the Eastman Dental Hospital (EDH). This screening procedure could have been performed on a potential participant more than once due to a previous visit not fulfilling the criteria of symptoms score, biopsy result, or antibiotic treatment. The nature of the disease meant that symptoms can change between their visits, which were ~6 months apart. Figure 1 shows that in total there were 1,748 OLP visits that were screened using the NHS CDR system over the period of the recruitment period (12 months). Around 79% (1,393 events) were excluded due to a
violation of the inclusion criteria. 355 subjects (21%) were identified as eligible based on the last correspondence letter generated by clinician who examine them. The CDR-eligible patients were then approached during their regular appointment and supplied with the study information. In this step 156 subjects (43.9%) declined the study information. Of the patients that received verbal and written information about the study (199 subjects), 26.6% (53 subjects) could not be contacted again and 55.8% (111 subjects) declined with some reasons such as full schedule, no symptoms, on/potential to use antibiotic, and concern about their tendency to vomit.

In the end, 35 potential participants were invited for the screening examination however 5 of them were then excluded due to having symptoms below the required level (4 subjects), or because they were on systemic antibiotics (1 subject). Participants were then allocated into the active and placebo group using 1:1 blocked randomisation method. All participants completed the 4 visits.

Thirty participants (24 women, 6 men) joined our study (Table 6.2). One participant from the placebo group was withdrawn from analysis due to antibiotic use for a urinary tract infection on day 13 of the trial, while two further participants were excluded from the VSL#3 group analysis due to a dental problem on day 11 and a urinary tract infection on day 14, which required antibiotics. In the final analysis, the placebo group had 14 participants with 11 women and 3 men and mean age 55.07 (SD=11.5). While the VSL#3 group had 13 participants with 10 women and 3 men and mean age 59.5 (SD=8.9) (Table 6.3). In terms of topical corticosteroid uses both groups have 10 and 11 participants for placebo and VSL#3, respectively. At baseline, participants in
the VSL#3 group reported a mean pain score of 5.23 (SD= 1.6), and the placebo group had a score of 5.39 (SD=2.1). There was no statistical difference in pain score between the two groups at baseline (p=0.757) Both groups showed the right and left buccal mucosa as the most frequent site with lesions (Table 6.4).

6.3.3 Acceptability and adverse event

One of secondary objective of the CABRIO study was to consider acceptability of the intervention and potential adverse events. Table 4 demonstrates that the active VSL#3 group showed the same proportion of associated-adverse events as the placebo group (3:3). As bloating is considered an expected effect of VSL#3, then we may draw the conclusion that probiotic VSL#3 is safe to consume for 30 days in the dosage of 2 sachets twice a day (one sachet contains a 450 billion probiotic cocktail). Thirty days consumption was decided based on minimum expected results from some studies. Table 3 also demonstrates acceptability of participant to consume the probiotic mixture for 30 days. In total participants have been given 128 sachets which were divided in two batches. The first 80 sachets were given at the baseline visit, 60 were supposed to be taken over the following 15 days and the 20 additional sachets were given to cover a 5 day window to accommodate any delays in attending the second visit. On the second visit, participants were given another 48 sachets and also had available the surplus from their first batch. Thus participants had 2 days or 8 sachets extra for the 30 days intake (120 sachets). Participants can consume their sachets by pouring it into their drink (not hot, or carbonised drink) or sprinkle it on their food (not hot). Thirty days consumption was choose based on average previous research, and assumption that the immunologic changing would appear after 30 days.
Percent of compliance was calculated as:

\[
\frac{(\text{Number of sachets given} - \text{number of left over sachets})}{\text{Number of sachets to be taken}} \times 100
\]

Four participants in the placebo group had more than 100% compliance, 6 had 100%, 3 had 90% and only 1 had 80%. Meanwhile, 6 participants in the VSL#3 group had over 100% compliance, none 100%, 5 had 90%, and 2 had 80% compliance. These data suggest that the probiotic mixture is acceptable to consume for 30 days in the dose of 2 sachets twice a day.
Figure 6.2 CABRIO participants recruitment.
Figure 6.3 CONSORT (Consolidated Standards of Reporting Trials) flow diagram. * screened event more than once, ** window time refer to ± 5 days flexibility of point visit.. AB= Antibiotic, CS= Corticosteroid
Table 6.2 Baseline demographic characteristics of CABRIO participants.  

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Group Placebo, n = 15</th>
<th>Group VSL3, n = 15</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean; SD) in year</td>
<td>56.13; 11.83</td>
<td>59.27 ± 8.25</td>
<td>0.397(^a)</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
<td>1.00(^b)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (80)</td>
<td>12 (80)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3 (20)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>Smoke (% Yes)</td>
<td>2 (13.33)</td>
<td>0 (0)</td>
<td>0.483(^b)</td>
</tr>
<tr>
<td>Alcohol (unit/week) (%)</td>
<td></td>
<td></td>
<td>0.940(^b)</td>
</tr>
<tr>
<td>0 – 5</td>
<td>13 (86.67)</td>
<td>11 (73.3)</td>
<td></td>
</tr>
<tr>
<td>6 – 10</td>
<td>1 (6.67)</td>
<td>4 (26.67)</td>
<td></td>
</tr>
<tr>
<td>11 - 15</td>
<td>1 (6.67)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Topical (%Yes)</td>
<td>11 (73.33)</td>
<td>12 (80)</td>
<td>0.657(^b)</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td>0.50(^b)</td>
</tr>
<tr>
<td>White</td>
<td>8 (56.67)</td>
<td>9 (60)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>7 (46.67)</td>
<td>6 (40)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3. Baseline demographic characteristic of CABRIO participants after exclusion due to antibiotic consumption * Fisher’s exact test

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Group Placebo, n = 14</th>
<th>Group VSL3, n = 13</th>
<th>p(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean; SD) in year</td>
<td>55.07 ± 11.5</td>
<td>59.5 ± 8.9</td>
<td>0.540</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
<td>11 (78.57)</td>
<td>10 (76.92)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3 (21.43)</td>
<td>3 (23.08)</td>
<td></td>
</tr>
<tr>
<td>Smoke (% Yes)</td>
<td>1 (7.14)</td>
<td>0 (0)</td>
<td>0.519</td>
</tr>
<tr>
<td>Alcohol (unit/week) (%)</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>0 – 5</td>
<td>12 (85.71)</td>
<td>10 (76.92)</td>
<td></td>
</tr>
<tr>
<td>6 – 10</td>
<td>1 (7.14)</td>
<td>3 (23.08)</td>
<td></td>
</tr>
<tr>
<td>11 - 15</td>
<td>1 (7.14)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Topical (%Yes)</td>
<td>10 (71.43)</td>
<td>11 (84.62)</td>
<td>0.287</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td>0.573</td>
</tr>
<tr>
<td>White</td>
<td>7 (50)</td>
<td>7 (53.85)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>7 (50)</td>
<td>6 (46.15)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.4 Baseline clinical characteristics of CABRO study, *Fisher's exact test

<table>
<thead>
<tr>
<th>Baseline variable</th>
<th>Group Placebo, n = 15</th>
<th>Group VSL#3, n = 15</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain score mean (± SD)</td>
<td>5.37 ± 2.04</td>
<td>5.4 ± 1.56</td>
<td>0.757</td>
</tr>
<tr>
<td>Site score mean (± SD)</td>
<td>8.8 ± 2.96</td>
<td>9.67 ± 3.35</td>
<td>0.385</td>
</tr>
<tr>
<td>Activity score mean(± SD)</td>
<td>13.07 ± 4.57</td>
<td>14.47 ± 6.47</td>
<td>0.49</td>
</tr>
<tr>
<td>ODSS mean (± SD)</td>
<td>27.23 ± 6.47</td>
<td>29.53 ± 9.37</td>
<td>0.891</td>
</tr>
<tr>
<td>Site lesion (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer lips</td>
<td>3 (20)</td>
<td>4 (26.67)</td>
<td></td>
</tr>
<tr>
<td>Inner lips</td>
<td>4 (26.67)</td>
<td>4 (26.67)</td>
<td></td>
</tr>
<tr>
<td>Right buccal mucosa</td>
<td>13 (86.67)</td>
<td>13 (86.67)</td>
<td></td>
</tr>
<tr>
<td>Left buccal mucosa</td>
<td>14 (93.33)</td>
<td>12 (80)</td>
<td></td>
</tr>
<tr>
<td>Gingiva lower right</td>
<td>10 (66.67)</td>
<td>12 (80)</td>
<td></td>
</tr>
<tr>
<td>Gingiva lower central</td>
<td>9 (60)</td>
<td>10 (66.67)</td>
<td></td>
</tr>
<tr>
<td>Gingiva lower left</td>
<td>10 (66.67)</td>
<td>13 (86.67)</td>
<td></td>
</tr>
<tr>
<td>Gingiva upper right</td>
<td>12 (80)</td>
<td>13 (86.67)</td>
<td></td>
</tr>
<tr>
<td>Gingiva upper central</td>
<td>12 (80)</td>
<td>11 (73.33)</td>
<td></td>
</tr>
<tr>
<td>Gingiva upper left</td>
<td>12 (80)</td>
<td>14 (93.33)</td>
<td></td>
</tr>
<tr>
<td>Dorsum tongue</td>
<td>2 (13.34)</td>
<td>1 (6.67)</td>
<td></td>
</tr>
<tr>
<td>Right ventral tongue</td>
<td>6 (40)</td>
<td>4 (26.67)</td>
<td></td>
</tr>
<tr>
<td>Left ventral tongue</td>
<td>4 (26.67)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>Floor of the mouth</td>
<td>2 (13.34)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>Hard palate</td>
<td>5 (33.33)</td>
<td>6 (40)</td>
<td></td>
</tr>
<tr>
<td>Soft palate</td>
<td>4 (26.67)</td>
<td>4 (26.67)</td>
<td></td>
</tr>
<tr>
<td>Oropharynx</td>
<td>1 (6.67)</td>
<td>2 (13.34)</td>
<td></td>
</tr>
</tbody>
</table>
6.3.4 Clinical variable analysis

6.3.4.1 Primary outcome: pNRS

The normal plots given in figure 6.4 show that the pain score data for all visits are approximately normally distributed, hence we could use the mean values for analysis. Average pain scores in the placebo and VSL#3 groups were, similar at baseline and both groups demonstrated a similar reduction at the day 30 - our primary endpoint) (Figure 6.5). Linear regression of the 30 day endpoint score adjusting for baseline showed no evidence of an effect of VSL#3 compared with placebo ($p = 0.789$, adjusted difference in mean pain score was 0.48 with 95% CI -1.46 to 1.12). The p value of 0.789 shows there is no evidence of difference in pain scores between the placebo and VSL#3 groups.

![Normal plots of variable pNRS at A. Baseline, B. 15, C. 30, D. 60-day endpoints](image)

*Figure 6.4 Normal plots of variable pNRS at A. Baseline, B. 15, C. 30, D. 60-day endpoints*
Based on this model, the coefficient of 0.48 for the comparison of groups indicates that on average mean pain score at 30 days is 0.48 units higher on VSL3 than on placebo, after adjusting for baseline score.

Both graph A and B in figure 6.5 show the mean pain scores at each follow up point and demonstrate that based on a regression analysis (adjusting for baseline) there is no evidence of a difference between the active and placebo group at any time point.
Table 6.5. List of associated and non-associated adverse events, and percent of compliance with expected sachet schedule for CABRIO participants

<table>
<thead>
<tr>
<th>Group</th>
<th>No</th>
<th>Study ID</th>
<th>Left over sachets</th>
<th>% Compliance*</th>
<th>Adverse Event</th>
<th>Associated</th>
<th>Non-associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>1</td>
<td>CABO-01</td>
<td>8</td>
<td>100</td>
<td>Shoulder pain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CABO-03</td>
<td>74</td>
<td>-</td>
<td>Withdrawn from study due to antibiotic use (UTI)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CABO-05</td>
<td>6</td>
<td>101</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CABO-09</td>
<td>8</td>
<td>100</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>CABO-10</td>
<td>8</td>
<td>100</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>CABO-12</td>
<td>4</td>
<td>103</td>
<td>Bloating</td>
<td>Cold</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>CABO-14</td>
<td>8</td>
<td>100</td>
<td>Cold</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>CABO-16</td>
<td>0</td>
<td>106</td>
<td>Nausea</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>CABO-18</td>
<td>8</td>
<td>100</td>
<td>Bloating/Nausea</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>CABO-20</td>
<td>14</td>
<td>95</td>
<td>Sinusitis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>CABO-22</td>
<td>30</td>
<td>81</td>
<td>Flatulence</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>CABO-29</td>
<td>4</td>
<td>103</td>
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* >100% compliance achieved when participant consumed product investigational more than 120 sachets which happen when participants came at visit 3 on plus windows time, i.e., more than 30 days.
**Figure 6.5.** Graph of mean pain scores A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of the same pain score calculated by subtracting each score from the baseline score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.4.2 Secondary outcomes

6.3.4.2.1 Oral disease severity score (ODSS)

The normal plots in figure 6.6 show ODSS data for all visits were approximately normally distributed, hence we decided to analyse means. For this outcome, average ODSS score in placebo and active groups were similar at baseline. The linear regression model comparing randomised groups with adjustment for baseline, showed that VSL#3 at the 30 day point had no evidence of difference in term of ODSS reduction ($p = 0.916$, adjusted difference in mean ODSS =0.69 with 95% CI -4.8 to 5.32). Results at the 60 day endpoint were similar ($p = 0.856$).

Although there was no evidence of difference between active and placebo groups, however figure 6.7.B demonstrated that at both 30 and 60 days endpoint the severity score for active group had more positive response.
(defined as bigger reduction in score adjusted from baseline) compare to the placebo.

**6.3.4.2.2 Activity score**

![Figure 6.8 Normal plots of variable Activity score. A Baseline, B. 15 2, C. 30 and 3, D. 60 day endpoints.](image)

Figure 6.8 demonstrated activity score data which were approximately normally distributed, we analyse this data by means comparison.

For this outcome, average site score in placebo and active groups were similar at baseline. The linear regression model comparing randomised groups with adjustment for baseline, showed that VSL#3 at the 30-day point had no evidence of difference in term of Site score reduction ($p = 0.896$, adjusted difference in mean Site score =0.58 with 95% CI -3.45 to 3.03). Results at the 60-day endpoint were also similar between groups ($p = 0.651$).
Figure 6.7 Graph of mean ODSS. A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of ODSS calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test. Positive and negative values were considered as worsening and improvement, respectively.
Figure 6.9 Graph of means Activity score A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of Activity score calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test) Positive and negative values were considered as worsening and improvement, respectively.
Although there was no evidence of difference between active and placebo groups, however figure 6.9.B demonstrated that at both 30 and 60 days endpoint the severity score of active group had more positive response (defined as bigger reduction in score adjusted from baseline) compare to the placebo.

6.3.4.2.3 Site score

The normal plots in figure 6.10 show site score data for all visits were approximately normally distributed, hence we decided to analyse means.

For this outcome, average Site score in placebo and active groups were similar at baseline. The linear regression model comparing randomised groups with adjustment for baseline, showed that VSL#3 at the 30-day point had no evidence of difference in term of Site score reduction ($p = 0.651$, adjusted difference in mean Site score $=0.67$ with 95% CI -1.49 to 2.34). Results at the 60-day endpoint were similar ($p = 0.976$).

There was no evidence of difference between active and placebo groups, however figure 6.11.B demonstrated that at both 30 and 60 days endpoint the severity score of active group had more positive response (defined as bigger reduction in score adjusted from baseline) compare to the placebo.
Figure 6.10 Normal plots of variable Site score. A Baseline, B. 15 2, C. 30 and 3, D. 60 day endpoints.
Figure 6.11. Graph of mean Site score A. at each follow up time point (P-values from analysis adjusted for baseline). B. mean reduction of Site score calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.4.2.4 Activity and Site scores

The normal plots in figure 6.12 show activity and site score data for all visits were approximately normally distributed. The data analysed by means comparison.

Average of Activity and site scores in placebo and active groups were similar at baseline. The linear regression model comparing randomised groups with adjustment for baseline, showed that VSL#3 at the 30-day point had no evidence of difference in term of both Activity and site scores reduction ($p = 0.906$, adjusted difference in mean Site score =0.64 with 95% CI -4.54 to 5.09). Results at the 60-day endpoint were similar between groups ($p = 0.712$).

Figure 6.13.B demonstrated that at both 30 and 60 days endpoints the severity score of the active group had a more positive response (defined as bigger reduction in score adjusted from baseline) compare to the placebo.

![Figure 6.12 Normal plots of variable site score and activity scores. A Baseline, B. 15 2, C. 30 and 3, D. 60-day endpoints.](image)
Figure 6.13 Graph of mean Activity and site scores A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of Activity and site scores calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.4.3 Quality of Life (QoL)

The normal plots in figure 6.14 represent QoL data for all visits were approximately normally distributed. The data analysed by means comparison.

Average of quality-of-life scores in placebo and active groups were similar at baseline. The linear regression model comparing randomised groups with adjustment for baseline, showed that VSL#3 at the 30 day had no evidence of a difference in term of QoL scores compared to placebo ($p = 0.587$, adjusted difference in mean Site score = 1.12 with 95% CI -6.78 to 10). Results at the 60-day endpoint were similar ($p = 0.983$).

Figure 6.15B however clinically demonstrated that at both 30 and 60 days endpoint the severity score of the active group had on average a more positive response on QoL (defined as bigger reduction in score adjusted from baseline) compare to the placebo.
Figure 6.15 Graph of mean Quality of life (QoL) score A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of Activity and site scores calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5 Mechanistic study

6.3.5.1 CXCL8 chemokine level

Figure 6.16 demonstrated CXCL8 chemokine serum level in pg/ml normal plots. All data visits have distribution that is skewed to the right with one outlier. Some of the participant’s cytokines level were below detection range and gave a zero number, hence we chose to use median for analysis.

![Figure 6.16 Normal plots of variable CXCL8 serum cytokine level at pg/ml A Baseline, B. 15 2, C. 30 and 3, D. 60 day endpoints.](image)

The linear regression model on figure 6.17.A revealed that at 30 days, the active group demonstrated no evidence of differences in the CXCL8 serum level with \( p = 0.874 \), adjusted difference in mean CXCL8 serum level was 1.1 with CI 95% -5.7 to 6.47. However, the mean reduction in CXCL8 serum levels were more pronounced in the VSL#3 arm than the placebo at day 30 when corrected for baseline levels (figure 6.17B), which suggests that there may be an effect of VSL#3 on CXCL8 serum levels.
Figure 6.17 Graph of median CXCL8 chemokine level, A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of CXCL8 level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.2 CXCL10 serum chemokine level

Figure 6.18 demonstrated CXCL10 chemokine serum level in pg/ml normal plots. All data visits have approximately normal distribution, hence means uses to analyse data.

Average CXCL10 in the placebo and VSL#3 groups were, as expected, similar at baseline and were similarly reduced by the 2-month visit ((Figure 6.19.A). Linear regression of the 30-day endpoint score adjusting for baseline showed no evidence of an effect of VSL#3 compared with placebo ($p = 0.141$, adjusted difference in mean pain score was 0.31 with 95% CI -0.86 to 5.67). The p value of 0.141 shows there is weak evidence of difference in CXCL10 serum level between the placebo and VSL#3 groups.

Figure 6.19.B displayed graph of mean reduction of CXCL10 serum cytokines where the VSL#3 arm had more positive response, indicated by bigger reduction in cytokine level when adjusted for baseline, compare to placebo arm.
**Figure 6.19** Graph of mean CXCL10 chemokine level A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of CXCL10 level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.3 IFN-γ cytokine level

![Graphs showing IFN-γ cytokine level](image)

Figure 6.20 Normal plots show skewed to the right distribution of IFN-γ cytokine level at pg/ml. A Baseline, B. 15 2, C. 30 and 3, D. 60-day endpoints. D, E, and F are the normal plots after log transformation, respectively.

Figure 6.20 demonstrated IFN-γ cytokine serum level in pg/ml normal plots. All data visits have approximately normal distribution, hence means uses to analyse data.

Linear regression of the 30-day endpoint score adjusting for baseline showed no evidence of an effect of VSL#3 compared with placebo ($p = 0.131$, adjusted difference in mean pain score was 1.13 with 95% CI -.0.13 to 0.9). The p value of 0.131 shows there is weak evidence of difference in IFN-γ serum level between the placebo and VSL#3 groups.

Figure 6.21.B displayed graph of mean reduction of IFN-γ serum cytokines where the VSL#3 arm had more positive response, indicated by bigger reduction in cytokine level adjusted by subtraction from baseline, compare to placebo arm.
Figure 6.21 Graph of mean IFN-γ cytokine level after log transformation A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of IFN-γ level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.4 IL-6 cytokine level

For IL-6 serum level pg/ml normal plots (Figure 6.22) for all visits show a distribution that is skewed to the right with one outlier, hence we chose to use the geometric mean (GM) for analysis after log e-base transformation. The linear regression model revealed that at 30 days the VSL#3 group demonstrated no statistically significant effect on IL-6 level pg/ml with $p = 0.321$, adjusted difference in mean log pain score was 0.75 with CI -0.57 to 0.2. The geometric mean ratio of VSL#3 and placebo reveal 1.1 value with 95% CI 0.57 to 1.22.

Figure 6.22 Normal plots show skewed to the right distribution of IL-6 serum level at pg/ml variable with A. Baseline, B. at 30 days, C. 60 day endpoint. D, E, and F are the normal plots after log transformation, respectively.
Figure 6.23 Graph of mean IL-6 serum level A. at each time point, B. Mean reduction of IL-6 cytokine level calculated by subtracting each score from the initial score. Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.5 TNF-α cytokine level

Figure 6.24 demonstrated TNF-α serum level in pg/ml normal plots. All data visits have approximately normal distribution, hence means uses to analyse data.

![Normal plots of variable TNF-α cytokine level at pg/ml. A Baseline, B. 15 2, C. 30 and 3, D. 60-day endpoints.](image)

Linear regression of the 30-day endpoint score adjusting for baseline showed no evidence of an effect of VSL#3 compared with placebo ($p = 0.445$, adjusted difference in mean pain score was 0.38 with 95% CI -0.47 to 1.05). Figure 6.25.B displayed graph of mean reduction of TNF-α serum cytokines where the VSL#3 arm had more positive response, indicated by bigger reduction in cytokine level adjusted by subtraction from baseline, compare to placebo arm.
Figure 6.25  Graph of mean TNF-α cytokine level A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of TNF-α level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.6 CXCL10 saliva chemokine level

Figure 6.18 demonstrated CXCL10 saliva chemokine level in pg/ml normal plots. All data visits have approximately normal distribution, hence means uses to analyse data.

Figure 6.26 Normal plots of variable CXCL10 saliva chemokine level at pg/ml. A Baseline, B. 15 2, C. 30 and 3, D. 60-day endpoints.

Similar to CXCL10 serum level, the average of saliva CXCL10 level in the placebo and VSL#3 groups were, as expected, similar at baseline and were similarly reduced by the 2-month visit ((Figure 6.27. A). Linear regression of the 30-day endpoint score adjusting for baseline showed no evidence of an effect of VSL#3 compared with placebo ($p = 0.363$, adjusted difference in mean pain score was 0.15 with 95% CI -1.52 to 4).

Figure 6.27.B displayed graph of mean reduction of CXCL10 saliva level where the VSL#3 arm had more positive response, indicated by bigger reduction in cytokine level adjusted by subtraction from baseline, compare to placebo arm.
Figure 6.27 Graph of mean CXCL10 saliva chemokine level, A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of CXCL10 saliva level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.7 IFN-γ saliva cytokine level

For IFN-γ saliva cytokine level pg/ml normal plots (Figure 6.28) for all visits show a distribution that is skewed to the right with one outlier, hence we chose to use the geometric mean (GM) for analysis after log e-base transformation. The linear regression model revealed that at 30 days the VSL#3 group demonstrated no statistically significant effect on IFN-γ level pg/ml with \( p = 0.265 \), adjusted difference in mean log pain score was 1.68 with CI -0.0.6 to 0.2. The geometric mean ratio of VSL#3 and placebo reveal 1 value with 95% CI 0.0.6 to 0.2.

Interestingly, figure 6.29.B demonstrated more response to VSL#3 group compared to placebo group with \( p \) value of 0.082 which was showed evidence of VSL#3 more response on IFN-γ saliva level reduction.
Figure 6.29 Graph of mean IFN-γ saliva cytokine level after log transformation, A. at each follow up time point (P-values from analysis adjusted for baseline), B. mean reduction of IFN-γ saliva level calculated by subtracting each score from the initial score (P-values from unpaired, two-tails, Student t-test). Positive and negative values were considered as worsening and improvement, respectively.
6.3.5.8 IL-1β serum cytokine level

The level of IL-1β serum cytokine was too low to consider for analysis.

6.3.6 Unplanned analysis: Topical corticosteroid usage

It is interesting and worth to note that on unexpected analysis of corticosteroid usage, we found out that participant on VSL#3 group reduced mean topical application per week by almost 50% (figure 6.30), which showed evidence of VSL#3 effect on day 30 and day 60 (figure 6.24 D). The number of participant using topical application also reduced from 11 to 6, and this number maintain until day 60.

Figure 6.30 Different presentation of corticosteroid (CS) usage through the study period. A). Mean reduction of CS application per week B). Individual CS applications at 30 days endpoint, C). Number of participants using CS throughout follow up study, D). Table of participant number using CS and without CS application in both groups and Fisher’s test result intergroup. *Statistically significant different between group, P < 0.1
6.4 Discussion

To our best knowledge, this was the first study of polybiotics in the treatment of OLP to measure clinical symptoms and markers of inflammation. However, Keller et al. performed a randomized pilot study on probiotic effects on recurrent candidiasis in OLP patients. Their study involved 22 participants using *Lactobacillus reuteri* dissolve intraorally three times a day for 16 weeks. This study needed to be terminated early due to recruitment problems since only a few patients with symptomatic OLP as their requirement attended to the trial site. This contradicted our study where the recruitment finished 3 months earlier than planned. A part from a different inclusion and exclusion cases, I believe that participant’s enthusiasm to participate in this study was based on their willingness to get an alternative, natural treatment other than corticosteroid. This was evidence by some participants who declined to use topical corticosteroid even though they reported painful symptoms.

The Keller study showed no evidence of a difference in terms of candidiasis recurrence, the need for symptomatic treatment or OLP severity score. Although, the gingival index and the pain score were lower in the probiotic group. This was, again contrary to our findings where the pain score in the probiotic group seems higher compare to placebo, although there was no overall statistically difference. Pain is a subjective feeling based on the perception of individual patients and contributed to by many factors including emotional state or physiological conditions.

In term of other clinical variables we investigated, both ODSS and QoL, which showed no evidence of a differences between placebo and active groups. However, when considering the score reduction for both variables, the VSL#3 group demonstrated more response during the period of study. In fact, when analysed separately, both site and activity scores demonstrated a greater reduction compared
to placebo group. In support of a potential positive role for poly-biotics in clinical responses, a study by Jianget al used a combination of Bifidobacterium longum, Lactobacillus lactis, and Enterococcus faecium in a capsule form given 2 times a day and reported improved efficacy and reduce the toxicity of chemotherapeutic agents and radiotherapy in oral mucositis (21) They suggested that the effects of the probiotics were through the modulation of the bacterial composition of the intestine. It could also be associated with the effects of the VSL#3 on wound healing which I demonstrated in vitro and described in Chapter 4. The mechanism of how VSL#3 can aid in wound healing is still unclear and needs further investigation.

Interestingly, although the pain score in the active group showed no reduction, even a slight elevation, the QoL score demonstrated an improvement. When the scores were adjusted from baseline there was evidence to support a potential response of participants to VSL#3. These finding are similar to the improvement in QoL previously reported in a randomized, controlled trial on liver disease. (17) This study also concluded that when used as secondary prophylaxis on Hepaticencephalopathy (HE) it lead to a significant reduction in hospitalization over a 6-months period. Furthermore, the VSL#3 probiotic also ameliorate encephalopathy, decreased inflammatory markers, and liver function improvement. Finally, a meta-analysis study on the use of VSL#3 in irritable bowel syndrome concluded that 3 out of 5 studies include in this meta-analysis showed only weak evidence of QoL improvement and therefore larger trials to validated multi-strains probiotic on IBS were warranty.(42)

Overall, an enhancement in the QoL of patients might be associated with a reduction in disease activity and site scores, which reflect a reduction in inflammation and the size of the lesion area in the oral cavity. A larger study with a longer
consumption of VSL#3 is warranted in order to determine if this theory can be validated in patients with OLP.

A number of studies have recently investigated the potential of dysbiosis of oral microbiome in OLP, which may lead to an imbalance between pathogenic and commensal bacteria. The dysbiosis might explain altered Toll Like receptor expression in the lesions of OLP previously reported. For instance, an over expression of TLR4 within the lesions of OLP patients compared to a control group was reported. A significant enhanced level of soluble TLR4 was identified in unstimulated whole saliva of OLP patients, and a reduced expression of TLR2 and an increase in TLR4 has also been reported. Another study detected a high expression of TLR4 and also TLR9 in OLP epithelium. It is possible that the elevation in TLR4 and potentially TLR9 may facilitate the induction of a pro-inflammatory response when activated by their specific ligands, LPS and bacterial/viral DNA respectively. The elevation in the inflammatory response has been reported for OLP and supported by my current work described in Chapter 3.

CXCL10 along with CXCL8 are a chemokine that function as chemoattractants for leukocytes such as T lymphocytes, neutrophils and monocytes. These chemokines have been reported to be up-regulated in OLP patients both locally or systemically, which suggests that these chemokines may play role in OLP. Some authors have suggested that CXCL10 can be used as a marker for LP inflammatory status or as a potential therapeutic targeted to reverse inflammation. My screening results demonstrated that circulatory CXCL10 was up-regulated in OLP patients and showed a strong correlation with the severity of the lesion (Chapter 3 figure 3.4) These results seem to support the notion that levels of CXCL10 can act as a marker of disease activity and severity. Hormannsperger et al
used an intestinal epithelial cell (IEC) model to test the effects of VSL#3 on immune responses and reported that the prebiotic could induce post-translational degradation of the CXCL10 protein and hence reduce its secretion. Additionally, our in vitro results using the human monocytic leukaemia cell line THP-1 and human oral epithelial cells MOE1a cells supported VSL#3 potential to inhibit CXCL10 secretion in vitro.

In this study, CXCL10 serum level which is a protein induced by IFN-γ demonstrated a degree of down-regulation after 30 days probiotic consumption ($p = 0.141$). CXCL10 levels at day 60 resulted in an elevation.. Although statistically there was no evidence of a difference between the two arms, the trend in improvement response of the patients in the VSL#3 group was higher compared placebo. Furthermore, the same pattern displayed on CXCL10 saliva secretion were affected more on active group compare placebo group ($p = 0.141$). This down regulation might explain reduction on activity and site score of participants in active group which potentially could have reduced the recruitment of leukocytes trafficking to the lesional area reducing inflammation. These finding suggest that VSL#3 could reduce the levels of CXCL10 in patients with OLP and this may be of some clinical benefit if it was sustained for a prolonged period of time.

In addition to CXCL10, IFN-γ serum and saliva cytokine levels demonstrated small but reproducible reduction ($p = 0.133$ and $p = 0.082$, respectively) after 30 days VSL#3 administration, but again, the effect could not be preserve for the 30 days after the administration ceased. An elevation is IFN-γ has previously been linked with OLP and it is also a major inducer of CXCL10 gene expression. My results may suggest that the changes observed in CXCL10 is directly associated with the levels of IFN-γ. Further work will be needed to determine if there is a relationship between IFN-γ and CXCL10 in OLP and if this is linked to disease activity.
Another interesting, unexpected result was CS topical application usage. Both the number of topical CS application per week and the number of participants using CS during the study were reduced by almost 50 percent. It is tempting to suggest that this might be associated with reduction in lesional area and less inflammation after probiotic administration. This result advocated that probiotic consumption might be a better adjunct alternative for OLP lesions since the potential adverse effect of corticosteroid usage on systemic conditions. Corticosteroid is the gold standard therapy for OLP lesions with a topical preparations afford good management without serious side effects resulting from systemic administrations. However, although rare, topical corticosteroid may also increase the risk of patients with suppressing the hypothalamic-pituitary-adrenal axis and even producing Cushing syndrome, especially in pediatric and geriatric patients. The transcutaneous/mucosal penetration might increase the potential side effects of topical corticosteroid. Several cases have been reported regarding systemic toxicity following topical application for instance, Jinagal reporting marked typical Moon Face and centripetal fat deposition of fat especially in buccal mucosa, neck, and trunk areas of a 6 weeks old boy, after 6 weeks of 0.1% topical betamethasone increased from 8 times a day to 12 times a day after pupilloplasty. Furthermore, two cases of Cushing’s syndrome due to overuse of topical steroid in diaper area also reported for two infants after 2 and 5 months Dermovate® (Clobetasol 17-propionate 0.05%) to treat eruption in the diaper area.

The analysis of saliva metagenomics changes during the CABRIO study is currently in progress. The reason this data delayed in analysis was due to long queuing for the 16S ilumina sequencing at the ICH facility. The findings from this part of the study have therefore been omitted from the thesis.
In the oral environment, topical corticosteroids have increased the risks of oropharyngeal candidiasis which probably associated to local immunosuppression. Over, topical steroid application applied on damaged or exposed epithelial that act as a barrier to the topically administered steroids such as on erosive lichen planus lesion may enhance steroid absorption and the potential for adrenal suppression. Some cases documented adverse effect associated with topical corticosteroid include the development of moon face of a series of patients treated with 0.05% clobetasol propionate in aqueous solution. Additionally, the long term application of topical corticosteroid has been proposed to induce a higher incidence of remission of signs and symptoms on OLP lesion although there was no valid data supporting this theory. Doses, duration and class of topical corticosteroid used contributed greatly on associated adverse effect that may occur.

Most importantly, VSL#3, which is a highly concentrated probiotic generally acceptable, tolerable, and safe enough to be used by OLP patients at a dosage of 4 sachets a day. As our results displayed there was no unexpected events in both groups (apart from the violation of the exclusion criteria which lead to participants being withdrawn from the study) that resulted in anyone not completing the trial. We also record no associated adverse events apart from the expected ones, such as bloating. However, both group showed the same ratio of bloating event, which suggests that it may not be linked to the live bacteria but the carrier material (maltose, cornstarch, and anti-cracking agent silicon dioxide). These associated adverse event did result in the participant’s withdrawing from the study. The compliance variable achieved an excellent achievement score, with a number of participants taking more sachets than required (compliance > 100%). This suggest that the participant has no hesitation in consuming the probiotic or the placebo.
As a conclusion, VSL#3 a multi-strains, high-concentrated probiotic, when used as adjunct therapy for OLP patients, resulted in a small, but currently insignificant reduction in severity of lesion in general and specifically for less site and activity area, improvement in participants QoL, decreased pro-inflammatory cytokines in the saliva/serum, potential to reduce topical corticosteroid usage, without any significant adverse event. These findings provide encouraging data to support the potential use of probiotics in patients with OLP.

There were a number of limitation in our study which need to be acknowledged.

1. Limited number of participants.
2. There was no inter and intra-examiner cross check.
3. There was no defined score regarding clinical variable to determine better condition/improvement for OLP lesion.
4. There was no clear definition regarding ‘pain’.
5. Our study targeting oral enviroment as the main delivery effect of the probiotic while we used VSL#3 sacchets which targeting gut environment as its target. This will reduce the effectivity of the probiotics.

I would strongly suggest the design and conduct of a larger, multi-centre study, which would incorporate a more detailed research investigation in order to ascertain the benefits of probiotics for OLP patients.
CHAPTER 7
CONCLUSION

8.1 Conclusion

The current study uncovered the potential of the probiotic VSL#3 mixture to inhibit the anti-inflammatory response of both THP-1 and MOE1a cell lines. By itself the probiotic VSL#3 did not activate a pro-inflammatory response or result in the loss in viability even at very high concentrations. These results provided reassurances about the potential safety of this agent on the immune system and oral epithelium and as a possible therapeutic agent for oral inflammatory diseases.

An effort was made to validate and isolate the specific strains contained in VSL#3. By the end of the project 7 out of the reported 8 strains were identified, either by culture (S. thermophilus, L. paracasei, L. plantarum, and L acidophilus) or by DNA sequencing (B. brevis and B. longus). B. infantis which were not isolated or identified might have been identified as B. longum since both species recently re-classified as B. animalis subs lactis due to them differing by only few single nucleotides.

VSL#3 3 seems to block at least two immune pathways (IFN-γ and TLR). The two pathways share some signalling components (NF-κB / IKK) and the VSL#3 could be working through an inhibitory receptor such as cytotoxic T-lymphocyte associated antigen 4 (CTL4) (see figure 8.1 and figure 8.2).

Patients with OLP were found to have a distinctive systemic cytokine profile with raised serum TNF-a, CXCL8, CXCL10, IL-6 and IFN-γ compared to HC. Within the saliva I was only able to identify CXCL10 as elevated in OLP and the levels correlated with the serum concentrations. These findings suggest that the local (saliva) and systemic levels of CXCL10 may be linked and associated with disease activity within the oral cavity. Additionally, IFN-γ which is an inducer of CXCL10
demonstrated a positive relationship for all clinical parameters analysed, specifically for pain. This data suggests that CXCL10 may be a reliable marker of disease activity in the saliva or blood and reducing IFN-γ and/or CXCL10 may provide some therapeutic benefit to the OLP patients.

Finally, our randomized double-blind, placebo, feasibility, two arms study demonstrated that VSL#3 mixture were generally acceptable, tolerable, and safe enough to be used by OLP participants at a dosage of 4 sachets a day. Furthermore, although not statistically significant, all clinical variables quantified except pain score demonstrated an improvement in the VSL#3 group compared to the placebo. In mechanistic study, probiotic VSL#3 could downregulate CXCL10/IFN-γ release from immune responsive cells such as tissue resident macrophages, Langerhans cells, T cells or keratinocytes resulting in a drop in local and systemic levels. The drop in pro-inflammatory cytokines will dampen the inflammatory tissue, increase the re-epithelisation of the oral mucosa, and might explain the improvements in ODSS and participant's quality of life. An interesting, unexpected result from this clinical study was CS topical application. Both the number of topical CS applications per week and the number of participants using CS during the study were reduced by almost 50 percent in the VSL#3 group. This reduction in CS use could be directly related to the anti-inflammatory effects of the probiotic and could be of significant clinical relevance to the patients. The results presented in the thesis provide supportive evidence for the use of probiotics as adjunct therapy in OLP. Further work is needed to support and validate the findings in patients and to unravel the mechanistic details of how probiotics suppress pro-inflammatory immune responses. If we can validate the effects of probiotics it could be revolutionary for the patients who suffer from OLP and potential to use as an alternative therapy for their lesions.
Figure 7.1 Suggested VSL#3 mechanism of action via inhibition of IFN-γ and TLR pathway.
8.2 Future work

The study results presented here suggest that the probiotic mixture VSL#3 has the potential to be an alternative treatment for OLP as an adjunct therapy along with corticosteroids. Due to the low numbers used in the pilot study the programme needs to be expanded further and additional mechanistic data is needed to identify the pathways/receptors responsible for the anti-inflammatory effects.

Studies to be followed up:

- Identifying and characterising the receptor which mediated communication between the probiotic and the cell host.
- Determine whether the raised IFN-\(\gamma\) and CXCL10 are linked to disease activity and if they are good biomarkers and therapeutic targets.
- Designing a larger, multi-centre study to confirm the effects of VSL#3 on OLP.

**Figure 7.2** Schematic negative modulation by VSL#3 poly-biotic in reducing inflammation via unknown inhibitory receptors. (picture adapted from Van Avondt K, et al, 2015.)
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REFERENCES


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Appendix 1 – Presentation

- The anti-inflammatory effects of the poly-probiotic VSL#3 on the oral mucosa: European Crohn’s and Colitis organization (ECCO) 15th congress, 12-15 February, 2020, Vienna, Austria.

- Probiotic mixture VSL#3 as an adjunct therapy for oral lichen planus: a double blind randomized study Eastman Research away day away poster presentation, May 2019.


- An interim analysis of CABRIO Study. Eastman student research day oral presentation, November 2017VSL#3 a highly concentrated probiotic accelerates wound healing, while maintain tight junction integrity in oral epithelial cells through an inhibition of pro-inflammatory cytokines release. Eastman Research away day poster presentation, May 2018.


- Do VSL#3 probiotic can be effective in treating oral lichen planus? UCL Infection, Immunology and Inflammation (III) symposium, London, November 2016.

- Student bursary award from IADR (International Association for Dental Research)-BSODR (British Society for Oral Dental Research) for IADR seminar meeting, London 2018
### Appendix 2 – Species found on 2 different populations

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<th>Species found only on Jewish population, n = 191</th>
<th>Species found on both population</th>
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Species found only on Jewish population, n = 191
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