Marine Algal Compounds as Novel Therapeutics for Food Allergy

Thesis submitted by

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Declaration

I hereby declare that the work conducted and presented in this thesis is my own work. The work carried out in this thesis has not been submitted in any other form for the award of another degree or diploma in another institution of tertiary education. The information discussed or derived from other published or unpublished sources has been duly acknowledged wherever required and referenced as per the guidelines. Studies conducted in collaborations has been acknowledged with the details of the collaborator or funding sources in both publication and within the compounds of thesis.

The research presented and reported in this thesis involving the use of animals was conducted in by following the guidelines provided by the James Cook University Animal Experimentation Ethics Review Committee. The relevant animal ethics approval numbers are provided as required within the chapters. A part of chapter-5 research work involved human serum samples, therefore, the relevant human ethics approval numbers associated with the sources of sample procurement (University of Melbourne) and MOU details are provided within the chapter.

Kunal Pratap

List of Publications

Publications included within the PhD thesis

Chapter 1

1. **Pratap K**, Taki AC, Johnston EB, Lopata AL, Kamath SD. A Comprehensive Review on Natural Bioactive Compounds and Probiotics as Potential Therapeutics in Food Allergy Treatment. *Front Immunol*. 2020;11:996. doi:10.3389/fimmu.2020.00996

Chapter 4

2. **Pratap K**, Majzoub ME, Taki AC, et al. The algal polysaccharide ulvan and carotenoid astaxanthin both positively modulate gut microbiota in mice. *Foods*. 2022;11(4):565. doi:10.3390/foods11040565

Chapter 5

3. Kabasser S, **Pratap K**, Kamath S, et al. Identification of vicilin, legumin and antimicrobial peptide 2a as macadamia nut allergens. *Food Chemistry*. 2022;370:131028. doi:10.1016/j.foodchem.2021.131028

Publications not included within the PhD thesis

1. Johnston EB, Kamath SD, Iyer SP, et al. Defining specific allergens for improved component-resolved diagnosis of shrimp allergy in adults. *Molecular Immunology*. 2019;112:330-337. doi:10.1016/j.molimm.2019.05.006

Table of Contents

List of Figures

List of Tables

Abstract

Food allergy is a type-1 hypersensitivity reaction mediated by IgE-antibodies upon ingestion or exposure to innocuous food proteins. Current incidences worldwide suggest that food allergy is an ever-growing health concern and predicted to rise further. Current preventative approaches are limited to avoidance of the causative food items. Treatment strategies for food allergy are limited to specific food allergen-based immunotherapies, with only one commercially available FDA approved immunotherapy for peanut allergy. Alternative approaches for preventing and treating different types of food allergy are currently needed to address the rising prevalence of food allergy.

This PhD thesis investigated the potential of marine green algae in suppressing an allergic response in an animal model of peanut allergy and analysed its effects on the gut microbiome.

In **Chapter 1**, a comprehensive, in-depth review of the currently known mechanisms of food allergy is presented, and the role of natural products such as marine algal compounds as potential therapeutics in food allergy treatment is discussed. Marine algae are a major untapped source of natural bioactive compounds and have been reported for their anti-allergic, anti-inflammatory, and immunomodulatory properties. In this review, we summarise the currently known mechanisms of food allergy and discuss the studies reporting the efficacy of natural bioactive compounds from different marine algae and their biological and functional relevance in modulating different types of food allergies. In the review, we also highlight the green algae *Ulva ohnoi*. This is the first study exploring the bioactive properties of isolated polysaccharides from *U. ohnoi* for their anti-allergic activities, focusing on peanut allergy. Furthermore, we also discuss the role of tree-nut allergens as cross-reactive sources for patients with peanut allergy.

In **Chapter 2,** a pilot trial was designed and conducted for evaluating the toxicity of the crude biomass and purified ulvan polysaccharide from green macroalgae *U. ohnoi*. No mortality and toxicity were observed after feeding the mice with crude biomass and purified ulvan over four weeks. Subsequently, a mouse model of peanut allergy was developed via intragastric sensitization using cholera toxin as an adjuvant. Crude biomass and purified ulvan were administered after establishing peanut allergy to

investigate their efficacy as anti-allergic compounds. In this study, a sustained peanut-based allergic response in the mouse model could not be achieved. Purified ulvan elicited a Th2 suppressing trend, but not statistically significant. In contrast, the crude biomass had a Th2 stimulating effect. In this study, several challenges and confounding factors were faced in the peanut allergy animal model design.

We suspected that the lack of a sustained allergic response in Chapter 2 could likely occur because of the length of immunization, choice of route of sensitisation, use of a specific pathogen-free facility or changes in the gut microbiota based on these factors. **Chapter 3** investigated the impact of these suspected factors by developing the mouse model of peanut allergy in two separate housing facilities (conventional and specific-pathogen-free [SPF]). The length of the immunization was reduced, and two different sensitisation protocols (intragastric and intraperitoneal) were implemented to evaluate the differences in the immune responses. Clinical and immunological presentation of an allergic response in the conventional facility was substantially stronger when compared to the SPF facility. Mice housed in SPF and conventional facilities showed a more prominent allergic response using intraperitoneal (I.P.) sensitization as compared to intragastric sensitization. In addition, an overall effect on bacterial diversity and community structures suggested significant differences between the SPF and conventional facility. In conclusion, various factors influence the establishment of a sustained peanut-based allergic response in a mouse model.

In addition, we needed to further explore the effects of feeding the purified ulvan on mouse gut microbiota. In **Chapter 4**, we investigated the impact of feeding the purified ulvan to mice on gut microbiota in parallel with a carotenoid (astaxanthin), widely consumed and studied for its prebiotic properties. Mice were fed with purified ulvan and astaxanthin for a similar period (28 days) as the model developed in Chapter-3. The study's objective was to investigate whether the changes in gut microbial diversity may skew towards tolerance or allergic sensitization. Purified ulvan and astaxanthin treatment enriched the overall gut microbiota with the commensal bacterial population, with *Bacteroidia*, *Bacilli*, *Clostridia*, and *Verrucomicrobia* found to be most abundant. This study established the safety and efficacy of purified ulvan and astaxanthin as potential prebiotic therapeutic candidates.

Peanut allergy is a widely studied type of food allergy because of its high incidence among children and adults and risk of cross-reactivity upon ingestion of other tree-nut allergens. **Chapter 5** discusses the risk of serum IgE reactivity in nut sensitized patient to macadamia nuts and other tree nut allergens. This study confirms the IgE reactivity of clinically confirmed peanut-allergic patient serum to macadamia nut extract and its purified allergens confirming the risk of potential cross-reactivity. Furthermore, IgE reactivity to different tree nut allergens was also assayed utilizing the grid blotting assay and ELISA. Clinically confirmed peanut allergic patient demonstrated higher IgE reactivity to other tree nut allergens in contrast to low IgE reactivity in clinically tolerant individuals.

In summary, crude and purified ulvan polysaccharide isolated from the green macroalgae *U. ohnoi* present no systemic toxicity and purified ulvan seem to suppress a peanut-specific immune response in mice. In addition, based on the optimisation of the caveats observed in allergy induction protocols and associated factors it was found that induction of an allergic response in mice is strongly dependent on the type of housing facility and route of sensitisation. Furthermore, purified polysaccharide feeding to mice has proven to enrich the mouse gut microbiota with commensal bacterial population and this could be explored further for therapeutic purpose. Lastly, the investigation of the clinical relevance of cross-reactivity of macadamia nut, its purified proteins and other tree nut allergens was established based on the IgE reactivity in clinically confirmed peanut allergic patient, verifying that there is a higher risk of clinical cross-reactivity in peanut allergic individuals.

The work conducted in this thesis increases the understanding of the safety and efficacy of novel marine algal compounds from *U. ohnoi* for their potential as alternative therapeutics or prebiotics, that can be further explored for other types of allergies. Future work involving macadamia nut and its purified proteins or tree-nuts as allergen source for developing a pre-clinical model can be conducted to investigate the efficacy of these compounds in mitigating the cross-reactivity between peanut and other nut allergens.

List of abbreviations

AD – Atopic Dermatitis

AIT – Allergen-specific immunotherapy

ALT – Alanine transaminase

AST – Aspartate aminotransferase

ASV – Amplicon sequence variants

APC- Antigen presenting cells

AK – Arginine kinase

BLAST – Basic Local Alignment Search Tool

BSA – Bovine serum albumin

CD – Cluster of differentiation

cDNA – Complementary deoxynucleic acid

CB – Crude ulvan biomass

CT – Cholera toxin

Da – Daltons

DC – Dendritic cells

°C – Degree Celsius

EpiPen – Epinephrine

ELISA – Enzyme-linked immunosorbent assay

EPIT- Epicutaneous immunotherapy

FALCPA- Food Allergen Labelling and Consumer Protection Act

FBS – Fetal bovine serum

FcεR – The high affinity immunoglobulin E receptor

HRP – Horseradish peroxidase

IFN – Interferon

IG – Intragastric

IgE – Immunoglobulin Isotype E

IgG – Immunoglobulin Isotype G

IgA – Immunoglobulin Isotype A

IL – Interleukin

IN – Intranasal

IP – Intraperitoneal

IV – Intravenous

kDa – Kilodaltons

LDH–Lactate dehydrogenase

l - Litre

LPS – Lipopolysaccharides

LNIT– Local nasal immunotherapy

MDA – Malondialdehyde

MHC – Major histocompatibility complex

MS – Mass spectrometry

MCP- Mast cell proteases

MLN – Mesenteric lymph node

µl – Microlitre

µM- Micromolar

ml – Millilitre

mg – Milligram

M – Molar

MLN – Mesenteric lymph node

mMCP-1 – Mouse mast cell protease-1

NA – Not applicable

ng – Nanogram

ns – Not significant

OAS – Oral allergy syndrome

OIT- Oral Immunotherapy

PAMP- Pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PERMANOVA – Permutational multivariate analysis of variance

pg – Picogram

PUFA – Polyunsaturated fatty acids

PGE2 – Prostaglandin E2

PU – Purified ulvan

RT-PCR – Reverse transcriptase – polymerase chain reaction

SC – Subcutaneous

SDS – Sodium dodecyl sulphate

SEM – The standard error of the mean

SCIT– Subcutaneous immunotherapy

SCFA- Short-chain fatty acids

SLIT – Sublingual immunotherapy

SOD – Superoxide dismutase

SPT – Skin prick test

SPF – Specific-pathogen-free

SLIT – Sublingual immunotherapy

TBS – Tris buffered saline

Th – T helper

TLR – Toll-like receptor

TNF- Tumour necrosis factor

Treg – T regulatory cells

Chapter-1

Marine algal compounds as a potential therapeutic for peanut allergy

In parts published in *Frontiers Immunology*, [10.3389/fimmu.2020.00996]

A comprehensive review on natural bioactive compounds and probiotics as potential therapeutics in food allergy treatment

Kunal Pratap, Aya C. Taki, Elecia B. Johnston, Andreas L. Lopata, Sandip Kamath

1. Allergy

Allergies are a severe health issue in developing and developed countries. Allergic diseases can cause significant distress to life and may be life-threatening in some cases [1,2]. The term allergy was coined by Clemens Von Pirquet, broadly defined as "*an altered capacity of the body to react to a foreign substance*". More specifically, allergy is now defined as an immune response upon exposure to an otherwise innocuous antigen and can also be termed hypersensitivity reaction [3].

The hypersensitivity reactions are classified into four major types based on their distinct immunological mechanism. The type I-III hypersensitivity reactions are antibody-mediated, whereas type-IV is a cell-mediated hypersensitive response. While the type I hypersensitive response is IgE mediated, IgG-mediated type II and III involve the Fc-receptor and complement-mediated effector mechanism [4]. Based on a hypersensitivity reaction's response time, these can be distinguished as immediate and delayed hypersensitivity reactions [4]. Immediate hypersensitivity reactions, as the name suggests, manifest early on due to the immune stimulus after allergen exposure, leading to an antigen-antibody complex. Delayed hypersensitivity reactions could take 1-3 days to manifest and are often mediated by T-cells [4].

Allergy is associated with type I hypersensitivity reaction which is mediated by IgE antibodies, also known as immediate-type hypersensitivity reactions [1,4]. The IgE antibodies specific to antigens are usually found tightly bound to the surfaces of mast-cells, eosinophils and basophils, the central effector cells in IgE-mediated type I hypersensitivity reactions, through the high-affinity IgE receptor known as FcεRI [1,4]. Interaction of antigen to IgE antibody induces a hypersensitivity reaction, cross-linking the receptors further causing the release of chemical mediators from the effector cells and leading to an allergic response [1,4] [Fig 1.1].

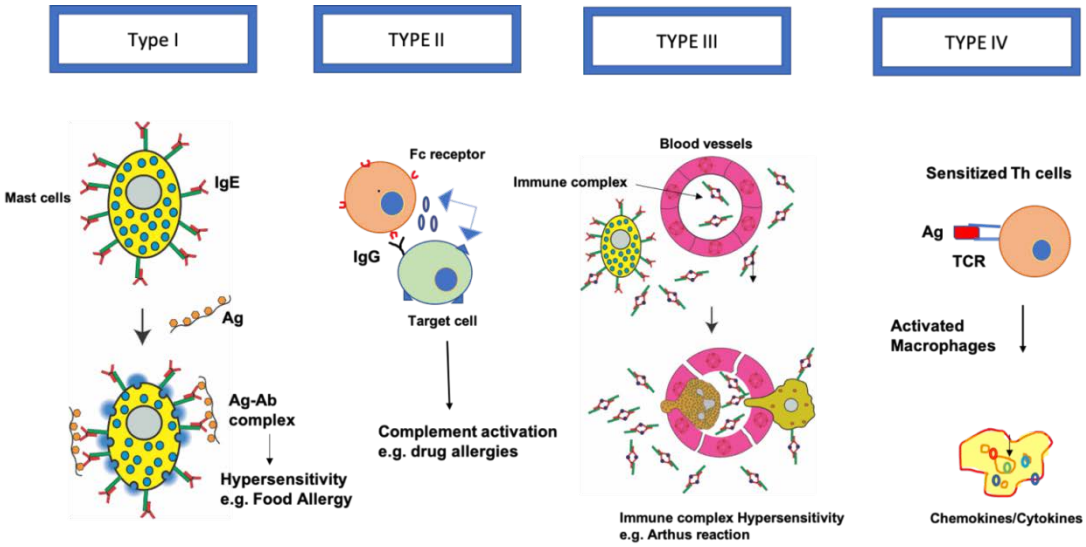

Fig 1.1: Different types of hypersensitivity reaction mediated by immunological mechanisms that cause tissue damage. [Adapted and modified from[5]]

1.1 Atopy

The term atopy is often used interchangeably with allergy; however, they are dissimilar. Atopy is the genetic tendency to develop an allergy [6,7]. An atopic individual exposed to allergens can develop an immune reaction leading to allergic inflammation, for example, food allergies, asthmatic response and dermatitis [6,7]. These allergic responses are often categorised as an amplified immune response towards allergens present in the environment and eventually lead to the production of allergen specific IgE. Various factors including genetic, host and environmental, influence the risk of allergen-specific IgE sensitization and can sometimes culminate in life-threatening conditions [8,9]. The atopy usually follows a particular pattern in the occurrence of symptoms and progression of the disease. The allergic condition usually starts with the development of atopic dermatitis (AD) and food allergy, then may be trailed by allergic asthma and rhinitis [10,11]. The incidence rate of allergic diseases has been increasing over the last few years, contributing to a high societal impact [12]. Among allergic reactions, food allergy-related pathologies are on the rise, for which the standard of care is still not optimal.

1.2 Mechanism of a Type-I hypersensitivity reaction

Type-I hypersensitivity is an acute reaction triggered by IgE antibodies generated against specific allergens [6,7]. A hypersensitivity reaction can lead to systemic or local

inflammatory responses, resulting in swelling, urticaria, eczema, airway hyper-responsiveness, asthma, and a life-threatening severe systemic response such as anaphylaxis [7].

A comprehensive overview of the mechanism of a typical type-I hypersensitivity reaction is presented in Fig 1.2. The sequences of events in the development of an allergic response begin with allergen presentation to the immune system via the gastrointestinal system, respiratory tract, or the skin, which leads to allergen specific IgE antibody production. This phase is termed 'allergic sensitization' [Fig 1.2]. IgE-dependent food allergies often manifest in infancy or early childhood; however, adult-onset food-allergies cases are increasingly recorded [13–15]. Mononuclear phagocytes in the gut and the Langerhans cells in the skin are central to food allergen's translocation across epithelial barriers. The sensitization phase involves allergen presentation to naïve $CD4^+$ T cells by antigen-presenting cells (APC) such as dendritic cells (DCs), resulting in the activation and differentiation of T cells into typically $CD4^+$, Th2 cells. Th2 cells, in turn, release cytokines, including interleukin-4 (IL-4), IL-5, and IL-13, which can promote the immunoglobulin class switch in B cells and differentiation into IgE secreting plasma cells [Fig 1.2]. The secreted antibodies bind to the FcεRI receptor on the surface of mast cells and basophils through its Fc region [16]. Two IgE receptors usually are present on different effector cells of the body, as FcεRI and FcεRII. The former is a high-affinity ($10^{10}\ M^{-1}$) cell-surface receptor that binds monomeric IgE molecule and is expressed on mast cells, basophils and dendritic cells [16]. The later FcεRII is a low-affinity IgE receptor expressed mainly on the monocytes, B cells, and dendritic cells[16]. The FcεRI receptor signalling is facilitated via a noncovalent interaction with a transmembrane dimer of low molecular weight called the Fc gamma chain (FcRγ)[16]. However, the FcεRII (CD23), low-affinity IgE receptor, in contrast, binds IgE with much lower affinity ($10^7\ M^{-1}$) than FcεRI and to a distinct region via its C_H3 domain[17,18]. Also, as opposed to FcεRII expressing a specific type of immunoglobulin, the cells that express FcεRI can possibly bind to variety of IgE antibodies with varying specificity.[18]. Thus, cross-linking of two IgE molecules with FcεRI is central to the induction and maintenance of an allergic response. In an allergic reaction, the FcεRIs are equipped to a greater degree with specific IgE, achieving a

concentration that permits cross-linking of the FcεRIs on exposure to the allergen and results in the production of allergen-specific antibodies.

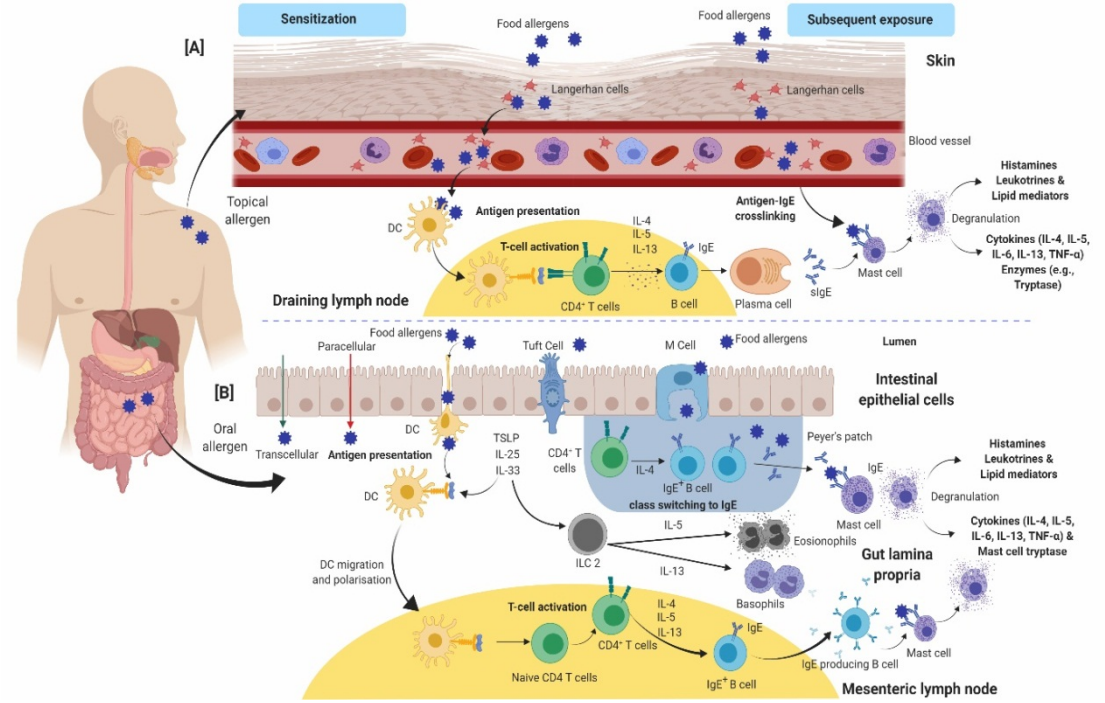

Fig 1.2: An overview of the immunological events occurring during allergic sensitization and effector phase upon exposure to food allergens via (A) skin and (B) gut. (A) In the epidermis, allergens are sampled by Langerhans cells, and the adaptive immune response is developed in draining lymph nodes. (B) In the gut lumen, allergens are taken up by DCs, and the subsequent events take place in Peyer's patches/mesenteric lymph nodes. Antigen-presenting cells (Langerhans cells in A or dendritic cells in B) present allergen-derived peptides to naïve CD4+ T-cells via MHC-class II complex. In susceptible individuals, naïve CD4+ T-cells polarize toward a Th2 phenotype and produce IL-4, IL-5, and IL-13. IL-4 and IL-13 induce the production of allergen-specific IgE antibody by B cells and clonal expansion. Allergen-specific IgE binds to FcεRI receptors on the surface of basophils and mast cells. On subsequent exposure to the same allergen (via contact or ingestion), the allergens bind, and cross-link cell bound IgE antibodies, which triggers degranulation and release of chemical mediators such as histamine, cytokines and prostaglandins. These mediators are responsible for the manifestation of an allergic reaction. Cytokines including IL-4, IL-5, IL-6, IL-13, and TNF-α are further released, which leads to cell-mediated late-phase allergic reactions through recruitment of eosinophils and Th2-cells. *CD, Cluster of differentiation; DC's, Dendritic cells; FcεRI, High-affinity immunoglobulin E receptor; MHC, Major histocompatibility complex; Th2, T-helper-2; IL, Interleukin; IFN-γ, Interferon γ; TNF-α, Tumour necrosis factor-α.*

Subsequent exposure to identical or similar allergens leads to binding and cross-linking two or more cell surface bound IgE antibodies. Allergen-induced IgE cross-linking triggers biochemical signals, leading to cell degranulation and secretion of lipid mediators, as well as the release of Th2 promoting cytokines [19–22] [Fig 1.2]. The

majority of mediators released during degranulation include vasoactive amines, lipids, cytokines, and proteases responsible for manifesting typical symptoms of an allergic reaction. Histamine is an early phase mediator of an allergic reaction that causes vasodilation, increased vascular permeability, and smooth muscles contraction. Like mast cell proteases (MCP), the release of proteases may cause local tissue damage contributing to inflammatory conditions including asthma [19,20,23]. Prostaglandins and leukotrienes follow suit and have a very similar effect on smooth muscles and vascular dilation [24]. Cytokines initiate the late phase reaction by recruiting leukocytes such as eosinophils, neutrophils, and Th2 cells. Mast cell degranulation activates tumour necrosis factor (TNF) release and IL-4, promoting inflammation by attracting neutrophils and eosinophils in multiple sites. On-site eosinophils and neutrophils can release proteases leading to localized tissue damage (e.g. eosinophilic esophagitis) [6]. Th2 cells may exacerbate the reaction by producing IL-5, recruiting more eosinophils to tissue sites and causing tissue injury [6] [Fig 1.2]. The abovementioned summary of the mechanism of action provides an overarching explanation of a general type-I hypersensitivity reaction. However, the allergen presentation and, subsequently, an allergic reaction can differ widely amongst individuals, increasing the complexity in understanding the exact pathophysiology behind allergic diseases, especially food allergies.

1.3 Allergens: Proteins and antigens

Individuals with allergic disease are either sensitized or predisposed (atopic) to produce IgE antibody-mediated response against some common environmental allergens, as listed in the presented table [Table 1.1]. Most allergens are highly soluble proteins or glycoproteins with multiple epitope sites that bind to IgE antibodies and instigate an early allergic response [25]. Certain proteins break down and form smaller subunits, which may cause an allergic reaction and are known as antigens [25]. In recent years, researchers have provided clues on the possible biological factors that make certain specific proteins allergenic [26]. The three distinct activities, namely, intrinsic enzymatic potential, production of molecules known as potential pathogen-associated molecular patterns, or PAMPS and IgE class switching upon subsequent exposure of allergens, are

heavily implicated in orchestrating an allergic response in predisposed or susceptible allergic individuals [26].

Table 1.1: Common allergens reported for type I hypersensitivity

Plant pollens	**Foods**	**Insect products**	**Drugs**	**Other allergens**
Ryegrass	Nuts	Bee venom	Penicillin	Animal hair and dander
Ragweed	Seafood	Wasp venom	Sulphonamides	Latex
Timothy grass	Eggs	Ant venom	Local anaesthetics	Mould spores
Birch tree	Peas, soybeans	Cockroaches	Salicylates	-
	Milk	Dust mites	-	-

The innate enzymatic activity (e.g. protease activity) of certain proteins found in pollen, cockroaches and dust mites has been reported to disrupt the intestinal epithelial barrier and the cell junctions leading to the absorption of these proteins by the innate cells and mounting an adaptive immune response [26]. In some specific cases, Der p 1 from house dust mites (*Dermatophagoides pteronyssinus)* could activate the complement system after the disruption in the mucosal barrier [27,28]. PAMPS, in some instances, are also known to mount an allergic response by interacting with the specific receptors of the innate immune system [29].

Lastly, in asthma and food-allergic responses, many allergens with low concentrations are introduced in individuals via mucosal tissue leading to the mounting of Th2 responses [7,30]. IL-4 and IL-13 are primarily produced by Th2 cells upon subsequent exposure, leading to heavy-chain class switching to IgE and plasma cells production, producing allergen-specific antibodies and generating a memory B-cell response [22].

1.4 Major food allergens

According to FALCPA (Food Allergen Labelling and Consumer Protection Act), eight well-characterized allergens must be declared on the packaging as per the regulations. These allergenic foods are commonly referred to as the Big 8 and include milk/dairy products, eggs, fish, crustacean shellfish, tree nuts, peanuts (legume), wheat, and soy [31]. The regulatory agencies have acknowledged the requisite to focus allergen labelling

rules on a regulated set of priority allergens. According to the FALCPA 2004, it is now mandatory to label the food with the ingredients if it has any major food allergen [31]. Among these Big eight allergens, peanut allergies are most common and are potentially life-threatening [32]. Though tree nuts and peanut belong to two distinct families of food, individuals with peanut allergies are often reported to be allergic to tree nuts [33]. However, other than these eight allergens, the allergic response against celery, cereals containing gluten and lupin are also reported [34].

1.5 Characteristics of food allergens

Proteins in food items are generally innocuous and hold nutritional value for human consumption. However, food allergenic proteins, despite being harmless, can elicit an allergic reaction. So, what makes these proteins allergenic? In recent times various distinct biochemical characteristics have been identified that can be associated with proteins identified as allergens. The most prominent features identified so far but not limited to are associated with the overall abundance of proteins in the food source, solubility, resistance to digestion and processing, glycation of proteins, IgE binding epitopes and structural homology amongst different allergens[35,36]. In general, food allergens are characterized as water-soluble glycoproteins that are relatively stable to acid digestion, heat and proteolytic activity, and small molecular size (10 to 70 kDa) [37]. The transfer and survival of the allergenic proteins across gastrointestinal tracts is ensured by their size, solubility, and stability, whereas in some cases glycation could also affect the overall stability and immunogenicity [38–40]. Apart from these prominent characteristics, intramolecular disulphide bonds and ligand-binding ability of certain proteins can also contribute to allergenicity[41]. For example, disulphide bonds provide stability against heat denaturation and enzymatic degradation to stabilize the allergenic proteins [41].

The sequence similarity (>70%) and similar structural moieties not only contribute towards the allergenic nature of the food proteins and contribute towards the cross-reactivity [42,43]. Some of the notable cross-reactivities reported include milk (4-92%), shellfish (75%), fish (50%), tree nuts (37%), grains (20%) and legumes (5%) [44].

In addition, structural and sequence similarities shared by different allergens have also revealed another critical determinant that contributes to overall immunoreactivity, i.e., epitopes. Epitopes are a protein portion that shares amino acid similarity between different proteins and henceforth can be recognized by antibodies or T cells [45]. Epitopes can be distinguished based on the structure, such as epitopes that binds to antibody can be linear or conformational; however, T cell binding epitopes are only linear [45]. Confirmation epitopes are generally susceptible to heat and enzymatic degradation (gastrointestinal digestion) and often considered less important in food allergy. In contrast, linear epitopes are highly stable under conditions as mentioned earlier and hence are considered biologically relevant to study food allergy [45,46].Most characterized food allergens share these biochemical properties; however, not all food proteins with these properties can become an allergen. The reason for this ambiguity is the complex nature of an allergic reaction that is multifactorial (genetic predisposition, allergen presentation and elicitation of Th 2 response).

1.6 Overview of food allergy

Food allergy is a type-I hypersensitivity reaction caused by protein antigens found in various food sources, marked by elevated levels of IgE antibodies that can lead to potentially life-threatening clinical reactions. More than 90% of all allergic episodes are recorded against eight major food groups: peanut, tree nuts, milk, wheat, soy, egg, fish and shellfish [15,47–49]. A recent cross-sectional survey involving over 40,000 adults in the US showed that at least 10% are food allergic, with the most common food allergy being to shellfish followed by milk, peanut, tree nut, and fish [15]. Currently, strict and careful avoidance of the offending food item is considered the best approach for preventing accidental allergic reactions [50]. In case of accidental exposure and subsequent severe reaction, an epinephrine auto-injector (EpiPen) is the only life-saving option [50].

Food allergy is a term used when a specific immunological mechanism has been involved in allergy onset. Any adverse reaction to food that does not include an immune response is not considered under the umbrella term food allergy [4]. For instance, many metabolic disorders, intolerance for lactose, alcohol, or pharmacologically active food components are usually not considered allergies to food [8]. Metabolic disorders are

generally dependent on various host-related factors rather than depending on a series of immunological responses; for example, in the case of lactose intolerance, the likely cause could be lactase deficiency in the host [51].

In a typical food allergic reaction, extrinsic lifestyle, and environmental factors contribute majorly to the disease's manifestations [12]. The presence of microbial communities such as the relative abundance of *Bacteroidetes* and *clostridia* has been linked to food allergies [52]. Also, the occurrence of food allergy has shown to be associated with ethnicity [53]. In Australia, children with one or both parents of Asian origin have been shown to be at a higher risk of food allergies. Infants belonging to parents of East Asian ethnicity have a higher risk of developing food allergy compared with infants of non-East Asian [53]. However, children born in Asia but then subsequently migrated to Australia showed a lower risk of allergy than children with Asian mothers born in Australia [54]. In terms of host genetics, the prevalence of the disease is again varied, such as mutations for filaggrin (FLG) gene is associated with most of the food allergies associated with the population of Singapore, a country inhabited by a huge Chinese-origin population, [55] compared to only two dominant FLG null mutations noted in European countries [56]. Similarly, differences in the occurrence of food allergy related to ethnicity have been reported in New Zealand, where Pacific Islanders shows higher chances of developing anaphylaxis as compared to other ethnic groups, and in the U.S., the African American children have higher rates of food allergy than White Americans [57]. These data extrapolate the development of food allergies in Asia and Africa in the recent decades due to increased urbanisation and adoption of a westernised lifestyle in such countries [12]. It is likely that additional factors might also play a role in food allergies such as food preparation, antacids, and exposure to skin creams containing food allergens such as almonds or peanuts [58]. In most developed countries, exposure of infant gut later in life with unintroduced food has also been hypothesised to play a role in the increase of food allergy [58].

1.7 Prevalence of food allergy

Allergic diseases are a global health issue posing a significant social and economic burden and reducing the quality of life [48,59]. Every year food allergy alone costs more than USD 24

billion to the U.S. economy, and a recent systematic review estimated a much higher economic burden at the household-level [59,60]. Prevalence-based studies have reported an alarming increase in food allergy in recent years, especially among children, reaching as high as 10% [47,48,50]. For example, in Europe and the United States, food allergy cases have been reported in 8-11% of the children and adult population [15,61–64]. Though estimating the exact widespread of food allergies is challenging as the controlled food challenge strategies are the gold standard for such estimations and can only be performed in specialized centres. However, few studies have attempted to measure food allergy prevalence; still, the estimate of the actual number is unattainable. Besides, inconsistencies in reporting the defined allergy have made the general population assessment extremely difficult, but the overall consensus noted that prevalence had been steadily rising [7]. Most of the population-based reports assessing the incidence of food allergy (not anaphylaxis) have majorly concentrated on peanut allergy, with increasing evidence in the United Kingdom and the United States [65]. Moreover, peanut allergy prevalence in children in the United States has doubled in the last few years, emphasizing environmental factors to play a role more than genetic factors. This could be explained as part of the hygiene hypothesis [65]. However, accurate assessments regarding the true incidence of food allergy are warranted to understand the rapid increase of the prevalence better.

In most cases, individuals will outgrow the disease naturally over time; however, the natural course majorly depends on the type of allergen itself. In most European countries as well as in Australia allergy from the cow milk and hen's egg are outgrown, approximately 2.5% of the newborn are diagnosed with cow milk allergy [51] and with the incidence of hen's egg allergy as high as 1.23% [62]. According to an estimate, the prevalence of peanut allergy is reported to double in next decade among children by 1.8%, 1.4%, and 3.0% in the developed nations such as United Kingdom, North America, and Australia respectively [53]. The prevalence of fish allergy in these countries varies from 0.1% to 7%, and that of shellfish allergy ranges from 0.3% to 10.3%; fish allergy seems more frequent in Asia than in Western countries [66]. There is still a need for more accurate prevalence and incidence data of allergy to specific food to educate consumers and health professionals about their food products that allow more transparency and informed choices to the individual.

1.8 Mouse models of food allergy: An overview

Animal models are indispensable in understanding the immunological mechanism of food allergy. Animal models have proven to be a reproducible portrayal of human physiology to establish new treatment strategies without risking human lives. Therefore, animal models' choice becomes crucial to investigate any mechanistic insights based on research goals.

Many animal species have been utilized in food allergy research, such as rat, dog, mouse, and swine [67]. However, the majority of preclinical research in food allergy research is centred around mouse models. Mouse food allergy models have been most widely accepted because of certain factors associated with mice, such as 1) their similar physiological and genetic architectures to humans, 2) shorter life span and low maintenance, 3) ease of genetic manipulation to disease-specific strains [67,68].

The typical setup of a mouse model of food allergy consists of three phases: sensitization of mice with the allergen of interest and the challenge phase to induce an allergic reaction and evaluation phase to measure physiological and immunological parameters [69]. A typical allergic response is dependent on the allergen uptake and subsequent presentation on the APC's using the MHC-II complex. However, mice have intrinsic oral tolerance towards these allergens and a disruption in the said tolerance is required for mounting an allergic response[67,69]. The most prominent method used for breaking the tolerance and developing a mouse model of food allergy is performed using adjuvants. Some of the most frequently used and reported adjuvants are cholera toxin (CT)[70–73], aluminium hydroxide (alum)[74–76], lipopolysaccharide (LPS)[77] and staphylococcus enterotoxin B (SEB)[78]. Conversely, adjuvant-free approaches where either the purified allergen or enriched extract is used to induce an allergic response have also been reported to be efficacious in mouse strains such as C3H/HeJ mice [reviewed in [79]]. The table below summarises allergy induction approaches used with or without adjuvants and their preferred routes of sensitization that are commonly used in food allergy research [Table 1.2].

Table 1.2: Table summarising adjuvanted and adjuvant-free approach with their preferred route of sensitization in various mouse models of food allergy.

Adjuvant	**Sensitization route**
Cholera toxin (CT) Staphylococcus enterotoxin B (SEB) Adjuvant-free	Oral gavage
Lipopolysaccharide (LPS) Aluminium hydroxide (Alum)	Intraperitoneal
Cholera toxin (CT) Lipopolysaccharide (LPS)	Intranasal
Adjuvant-free	Intradermal

Food allergy response in mice depends mainly on the genetic susceptibility of the strain [69]. As it is unfavourable to study any allergic repones directly into patients' samples due to significant heterogeneity, the mouse strains with the defined genetic background can be a plausible solution. The recent advancement in genetic manipulation research has led to the development of two distinct approaches, i.e., mouse models based on genetically modified strains of mouse[13,80,81] and humanized mouse models[82–85]. Genetically modified mouse strains in food allergy research provide an avenue for introducing genetic changes to skew an allergic response towards Th2-based sensitization[81]. The genetic changes disrupts either the differentiation and function of Treg cells or facilitate a Th2 biased response[81]. IL-4RaF079 mice are genetically modified by inactivating the inhibitory ITIM motif of the IL-4 receptor alpha chain resulting in significantly increased production of allergen-specific IgE levels in animals that were sensitized with ovalbumin in conjunction with CT[13]. Furthermore, mice that were given ovalbumin without CT as adjuvant resulted in strong temperature drop and incidences of diarrhea after subsequent challenges[13]. Alternatively, humanized mouse models of food allergy are curated to utilize human B and T cells in producing allergen specific human IgE in an *in vivo* setting[84]. As an example, NSG mice are engrafted with the human hematopoietic stem cells specifically CD34+ cells in span of 4 month. The NSG

engrafted mice that were orally exposed to peanut for 8 weeks and upon challenge produced peanut-specific IgE and anaphylaxis[84]. In a separate study, a humanized mouse model was curated using isolated human used PBMCs combined with peanut antigens that were reconstituted in NSG mice and subsequently exposed to peanut allergen producing peanut-specific IgE[85]. Both genetically altered models and humanized mouse models highlight the possibility of studying food allergy *in vivo* by curating mimicking human immunological features.

However, apart from the vast similarities between human and mice, specific considerations towards the disparities between the two should be considered while interpreting the results for translation in humans [86]. Mice are not inherently predisposed for allergic diseases; hence they are artificially induced with an allergic response. The artificially induced allergic/anaphylactic reaction in mice could be due to IgG or Fcγ receptors, which is inherently possible in mice but not in humans [86–88]. Similarly, the most prominent receptor involved in mounting an allergic response is the FcεRI receptor reported to have unequal expression levels in mice (only expressed on basophils and mast cells) than humans [89,90]. Additionally, another critical aspect of an allergic response is B-cell isotype switching to IgE which does not happen in mice, leading to a diverging mast cell profile between an allergic reaction induced in mice and one presented in humans [89]. However, mouse models have remained a cornerstone in increasing our understanding of the pathophysiology of food allergy. With the development in genetic manipulation techniques and the characterisation of different genetic backgrounds in mouse strains, the gap between the differences in immunological response across humans and mice will be less for better understanding in future research.

1.9 Nut allergy: An ever-growing concern

Nuts have been an integral part of the human diet for a long time [91]. In broad terms, nuts can be defined as the kernels wrapped in solid shells utilized for their oils and consumed as food items [91]. In food allergy, the most common nut known to result in a life-threatening allergic reaction is a peanut. Peanut allergy is the most common nut

allergy and is also categorized under the Big-8 food products known to cause most food allergy instances [25]. Other types of nuts are broadly classified under the term "tree nuts", namely, hazelnut, Brazil nut, pistachio, almond, pecan, walnut and cashew [92]. The reported cases of reactions to tree nuts are not on par with the peanut allergy reactions; however, the prevalence has been reported to increase in the past decade at an alarming rate [92,93]. As opposed to peanut allergy, tree nut allergy is understudied, leading to the lack of knowledge on the prevention and management [93].

Peanut and tree nuts protein's biochemical properties have been conserved ever since they were introduced as a nutritional source in the human diet [33]. So, what is it that changed in the past decade? What could be the likely cause behind the sudden rise in peanut allergy cases globally? Answering these questions is not an easy task due to the variety of factors that have been implicated by researchers as mediators in the induction of nut allergies in humans. Factors such as a change in feeding practices and hygiene during the early stages of life have certainly played a significant role in sensitizing the individual through the skin and/or intestinal barrier disruption [50,93,94]. Factors can range from adopting a modern lifestyle to change in hygiene growing up and allergen introduction by various means, contributing to immune dysregulation and food allergy development.

1.10 Association between peanut and other nut allergens (cross-reactivity)

Peanuts and tree nut allergens are classified based on the protein superfamilies [33,93]. Peanut has eighteen (Ara h 1-18) allergens reported in IUIS/WHO allergen database [95]. The majority of shared allergens are classified under six superfamilies: cupin, prolamin, Bet v1 like, profiling, glycosly-transferase GT-C and Scorpion toxin-like knottin [96]. Allergens under these six protein superfamilies have different functions associated with them. Some are related to structural composition, some act as storage proteins, and some have roles in defence mechanisms [96]. The detailed list of allergens with other related information is composed in [Table 1.2].

Due to peanut and tree nut allergens' homologous nature, cross-reactivity amongst the nut allergens is very common [96]. Sensitization to multiple proteins from a similar

protein source is often associated with clinical presentations [97]. Cross-reactivity between peanut allergens, tree nut allergens and even other legumes are very common; however, the reaction's severity can vary [97]. It has been shown earlier that around 50% of peanut-allergic patients have been reported to be co-sensitized to other legumes but were found not to be allergic [98].

Peanut allergy is among the most commonly reported nut allergy worldwide, and most of the allergens are widely studied for their biochemical properties and structural integrity [32,97]. Biochemical analysis of the peanut allergens has confirmed that most of these proteins are resistant to the digestive process, increasing the likelihood of presenting these allergens to the immune system and instigating an allergic response [99–102]. Disulphide bonds present in these proteins make these proteins resistant to the low pH (digestion) and heat resistant [96,102]. The heat resistance of peanut protein is also significant when studying in allergy context as most plant proteins are prone to undergo Maillard reaction where free amines react with sugar entities to form advanced glycosylation end products [103]. As a result, when peanut is dry roasted for consumption purposes, it accelerates Maillard reaction [103,104]. It has been demonstrated that roasted peanut proteins have a high likelihood of binding to the IgE than the raw peanut proteins [104].

Table 1.3: Summary of peanut and tree allergens based on protein family and superfamily.

Protein Superfamily	**Cupin**		**Prolamin**		**Bet v 1-like**	**Profilin**	**Glycosyl transferase GT-C**	**Scorpion toxin-like knottin**
Protein Family	**Vicilin (7s globulin)**	**Legumin (11s globulin)**	**2s albumin**	**nsLTP**	**Bet v 1 family**	**Profilin**	**Oleosins**	**Plant defensin**
Allergen Source								
Peanut [105] (*Arachis hypogaea*)	Ara h 1	Ara h 3, 4	Ara h 2, 6, 7	Ara h 9, 16, 17	Ara h 8	Ara h 5	Ara h 10, 11, 14, 15	Ara h 12, 13
Hazelnut[106] (*Corylus avellana L.*)	Cor a 11	Cor a 9	Cor a 14	Cor a 8	Cor a 1	Cor a 2	Cor a 12, 13	-
Cashew nut [106] (*Anacardium occidentale*)	Ana o 1	Ana o 2	Ana o 3	-	-	-	-	-
Pistachio [106,107] (*Pistacia vera*)	Pis v 3	Pis v 2, 5	-	-	-	-	-	-
Walnut [106,108] (*Juglans*)	Jug r 2, 6	Jug r 4	Jug r 1	Jug r 3	Jug r 5	Jug r 7	-	-
Almond [106,109] (*Prunus dulcis*)	-	Pru du 6	-	Pru du 3	Pru du 1	Pru du 4	-	-
Pecan [106] (*Carya illinoinensis*)	Car i 2	Car i 4	Car i 1	-	-	-	-	-
Brazil nut [106] (*Bertholletia excelsa*)	-	Ber e 2	Ber e 1	-	-	-	-	-

Similarly, tree nut allergens from hazelnut (Cor a 9 and Cor a 14) are also documented to have similar stability towards heat and pH, making them responsible for more severe reaction in tree nut allergic individuals [106]. Proteins from oleosin, defensin and LTP family are also reported for clinically relevant allergic reactions [106]. Conversely, proteins from profilin family are heat and pH labile and hence, are responsible for less severe reactions possible due to the breakdown of allergens during the digestive process [106].

Peanut and tree nut cross-reactivity's clinical relevance is significant due to the shared protein structure and sequence similarities. Previously a study has indicated that the cross-reactivity of peanut-specific IgE with almond, Brazil nut and hazelnut is patient-centric, as reported using the inhibition ELISA's [110]. In another study with a patient cohort of 324 peanut-allergic patients, it was reported that 86% of the patients were sensitized to tree nut; however, only 34% were found to be allergic to tree nuts clinically [111]. A separate cohort of peanut-allergic patients has been reported to have cross-reactivity to various nuts, with more than 49% of these patients showing reactivity to hazelnut [112]. In line with these observations, another recent study has reported that peanut-specific IgE from peanut-allergic children has a certain amount of cross-reactivity to almond, hazelnut, and pistachio measured *in vitro* using reciprocal inhibition immunoblotting [113]. In estimate, 20-40% of the peanut-allergic individuals are likely to have co-allergy towards different types of tree nuts [93,114,115].

1.11 Current therapeutics and biologics for peanut allergy

Currently available treatment strategies involve allergen-specific immunotherapy (AIT), a specialized and targeted treatment procedure performed to induce tolerance in individuals against specific food allergens [116]. AIT exposes the allergic individual to small but increasing doses of the allergenic protein, resulting in desensitization or lowered allergen reactivity. The main goal of AIT is to achieve sustained immune unresponsiveness to the food allergen [50,116,117]. There are different routes by which AIT can be administered, such as SCIT (subcutaneous immunotherapy), SLIT (sublingual immunotherapy), OIT (Oral Immunotherapy), IDIT (intradermal

immunotherapy), EPIT (epicutaneous immunotherapy) LNIT (local nasal immunotherapy) and ILIT (intralymphatic immunotherapy) depending on the types of allergens [116,118]. OIT is currently one of the preferred ways of administering AIT for peanut, egg and milk allergy and has been reported to induce desensitization [119]. Recently, the Food and Drug Administration (FDA, USA) approved PALFORZIA [Peanut (*Arachis hypogaea*) Allergen Powder-dnfp, Aimmune Therapeutics] as an OIT and the first therapeutic available for the treatment of peanut allergy for patients aged 4 years through 17 years of age [120]. However, for other food allergen sources, there are currently no curative therapies available. AIT is currently the most researched and potential therapeutic approach for food allergy, which has a disease-modifying capacity.

Recently Pajno et al. published EAACI guidelines on AIT for IgE mediated food allergy, providing comprehensive information on the evidence-based dose recommendations for different types of AIT regimens in clinically diagnosed patients with food allergy [117]. EAACI guidelines further elaborate and discuss the safe implementation of existing immunotherapy, associated inherent issues and challenges based on medicinal and social outlook [117]. These natural sources may have the potential to complement current AIT-based approaches to provide tolerance against allergic diseases.

1.12 Marine algal compounds and their role in modulating food allergy

1.12.1 Marine algae

A wide variety of novel active metabolites from marine algae have been reported for their biological properties, particularly sulphated polysaccharides from various species of marine algae for their anti-inflammatory, anti-allergic, anti-coagulant and anti-oxidant activities [121–125]. Three major groups of marine algae: brown algae, red algae, and green algae, are the primary sources of sulphated polysaccharides. Several recent reports have indicated that chemically active metabolites from marine algae can suppress allergen-specific antibodies such as IgE, IgG, IgG1 by downregulating CD3,

CD4 and CD8 cell surface receptors, thereby attenuating the cytokine response (e.g. IL-4, IL-5, and IL-13) in both *in vitro* and *in vivo* models of allergy [74,126–129].

1.12.2 Polysaccharides: Natural active compounds

Polysaccharides are a heterogeneous group of macromolecules with various biological properties that can act as potential therapeutics for human diseases [130]. These macromolecules consist of large monomeric units of monosaccharides joined together by glycosidic linkages. Polysaccharides can be hydrolysed by acid hydrolysis or, with the aid of specific enzymes, to produce the monomeric monosaccharide units [131,132]. Based on the type of monomeric units, the polysaccharides can be distinguished as homopolysaccharides (homoglycans) or heteropolysaccharides (heteroglycans) [133]. Heteropolysaccharides may also contain non-carbohydrate units along with monosaccharides. In the human gut, Bacteroidetes, Firmicutes and Actinobacteria are some of the dominant phyla responsible for enzymatically degrading dietary polysaccharides and producing functional secondary metabolites such as short-chain fatty acids (SCFA's), butyrate's and mucin [130,134]. These dominant bacterial phyla's are responsible for secreting different classes of CAZymes (carbohydrate active enzymes) such as glycan utilizing glycoside hydrolases, carbohydrate esterase's, sulfatases and polysaccharide lyases, which are further classified and reported based on family members of respective enzymes in the CAZy database (www.cazy.org) [135,136]. Conversely, the human gastrointestinal system secretes a limited number of enzymes essential for digesting dietary fibre polysaccharides such as starch. Interestingly studies have reported the ability of geographically distinct populations that can catabolize marine algal polysaccharides such as alginate, carrageenan and porphyrans [137,138].

Different polysaccharides have been reported to contain sugar molecules, such as galactose, rhamnose, fucose, and arabinose, acting as immune potentiators; and reported for their anti-coagulant, anti-HIV and anti-oxidant activities [132,139–142]. Moreover, studies have also reported that polysaccharides can influence the immune response upon digestion by downregulating Th2 cytokines and suppressing allergic inflammatory responses in the gut [74,121,143,144].

1.12.3 Green algal compounds in modulating food allergy

Natural compounds have been an attractive source for the prevention or treatment of various immunological disorders. The efficacy of natural compounds has been extensively reported in past decades. The sources for these natural compounds range from polysaccharides from marine algae or non-algal origins to traditional medicinal systems such as TCM and medicinal plants. Here, we discuss the role and efficacy of natural compounds from marine algal sources in influencing allergic disorders, focusing on food allergy.

Functional components derived from green algae are reported to be efficacious for various diseases such as type-2 diabetes, colitis and hepatocellular carcinoma [145–150]. Reported studies indicate sulphated polysaccharides to be the most active component of green algae by modulating gut microbiota, gene expression levels and carcinogenesis in various mouse models [145,151]. Flavonoids and polyphenols extracted from green algae have been shown to regulate gene expression and gut microflora in type-2 diabetes mouse models [146,149]. Furthermore, alkaloids are shown to attenuate colitis in murine models [147,148]. A recent systematic review on ulvan, a cell wall polysaccharide commonly found in green algae, explored various functional components, toxicity, and biological significance, indicating that ulvan may prove beneficial for preventing allergic diseases [132]. In another study, it was reported that ulvans isolated from green algae *Ulva ohnoi* demonstrated no toxicity and only mild immunomodulatory properties through elevated levels of IL-10 and decreased levels of prostaglandin E2 (PGE2) in lipopolysaccharide (LPS) stimulated murine macrophages [132]. This study also reported that higher molecular weight components of the isolated fraction had elevated immunomodulatory response in LPS stimulated murine macrophages; however, a minor elevation in pro-inflammatory cytokines such as IL-6 and IL-1β was also noted [132].

Currently, limited research evidence demonstrates the efficacy of green algae *Ulva ohnoi* to attenuate allergic symptoms. However, some active functional components could be utilized against food allergy. Current evidence suggests a potential role of

polysaccharides isolated from *Ulva ohnoi* as a preventative measure for food allergy. A summarised overview of marine algae's mode of action, focusing on natural compounds that affect specific immunological signalling pathways, is presented in the figure below [Fig 1.3]. In brief, three major groups of marine algae: brown algae, red algae, and green algae, are the main sources of sulfated polysaccharides. Several recent reports have indicated that chemically active metabolites from marine algae can suppress allergen specific antibodies such as IgE, IgG, IgG1 by downregulating CD3, CD4, and CD8 cell surface receptors thereby attenuating the cytokine response (e.g., IL-4, IL-5, and IL-13), in both *in vitro* and *in vivo* models of allergy[74,127,128,130]. For example, fucoidan is a sulphated polysaccharide commonly found in different brown algae species. The anti-allergic activity of fucoidan from *Undaria pinnatifida* in ovalbumin-induced mouse airway hypersensitivity has been shown to suppress IL-4, IL-5, and IL-13 release and reduce concentrations of eosinophils in bronchoalveolar lavage[153]. Alternatively, a passive as well as active cutaneous anaphylaxis model of ovalbumin and shrimp allergy in Balb/c mice, brown macroalgae extracts from *Sargassum tenerrimum*, *Sargassum cervicorne*, and *Sargassum graminifolium (turn)*, when injected peritoneally, suppressed the anaphylactic response[129]. Based on these reports, intraperitoneal administration of polysaccharides seems to downregulate pro-inflammatory cytokines, IL-4, IL-5, and IL-13 levels, and suppressing the anaphylactic response through elevated production of IL-12 and IFN-γ. However, local administration of algal polysaccharides provides a significant challenge of extrapolating the required dose in humans which will be much higher as compared to the murine model [Fig 1.3]. Currently, limited research evidence is available demonstrating the efficacy of green algae to attenuate allergic symptoms. However, some active functional components could be utilized in future for further investigations against food allergy.

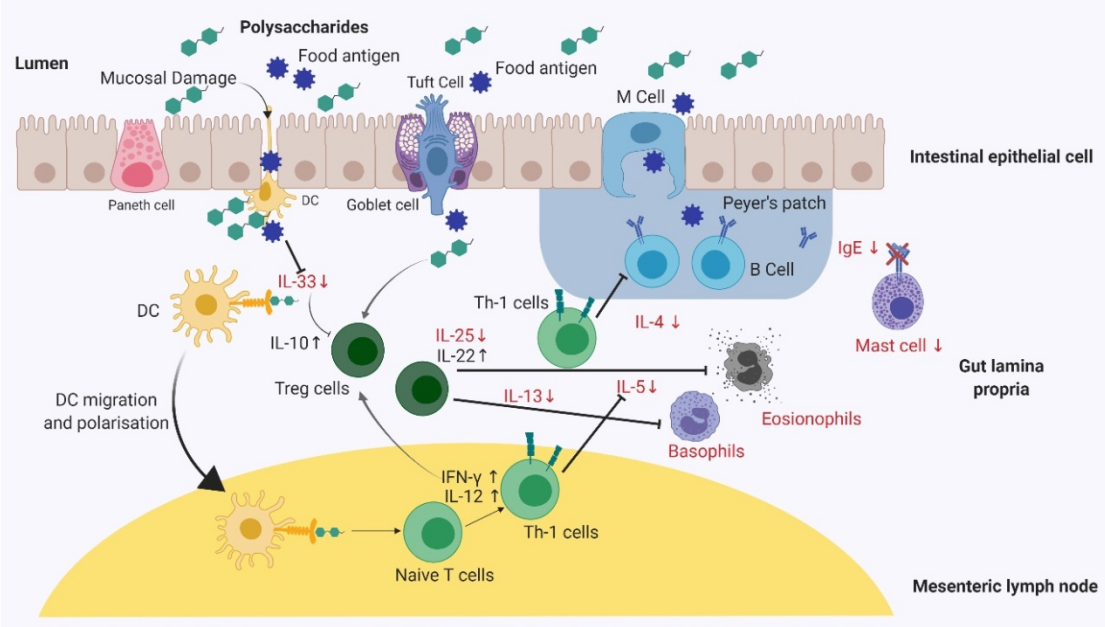

Fig 1.3: A graphical summary of the effects on different cell populations and cytokines involved in an allergic immune response after exposure to polysaccharides. *Th1, T-helper-1; DC's, Dendritic cells; IL, Interleukin; IFN-γ, Interferon-gamma.*

1.12.4 Green algae: *Ulva ohnoi* and its compounds

Ulva ohnoi (M. Hiraoka & S. Shimada) is a green macroalga belonging to the genus Ulva [152,153]. Recently, this species has been identified at various places and has been associated with green tide formation because of its rapid growth [152]. Recent studies have shown that *U. ohnoi* is rich in algal biomass, which can be utilized for high protein and polysaccharide yield [154]. Consequently, the industrial, aquacultural feeding and biological applications of these macroalgae are boundless. *U. ohnoi* has also been reported as functional dietary ingredients for feeding juvenile Senegalese sole (*Solea senegalensis*) [155] and phytoremediation properties by removing cadmium [156]. Ulvan polysaccharides are composed of monomeric disaccharide units, and these subunits have been reported to hold biological relevance as immunomodulatory compounds against several diseases [121,124,157–159]. Some of the disaccharide unit structures from *U. ohnoi* are presented below [Fig 1.4a, b, c, and d]

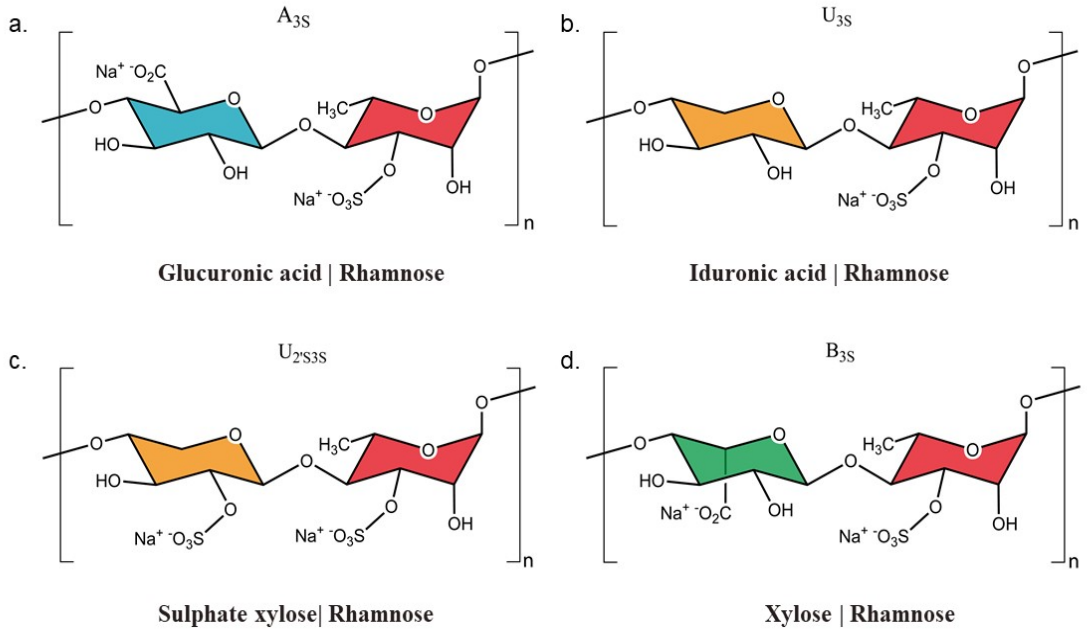

Fig 1.4: Structure of purified ulvan disaccharides from green macroalgae *Ulva ohnoi*. The polysaccharides in *U ohnoi* are composed of different monosaccharides such as a.) glucuronic acid, b.) iduronic acid, c.) sulphate xylose, and d.) xylose with the common monomeric unit of rhamnose.

1.13 Food allergy and microbiome

The role of the gut microbiome in the pathogenesis and progression of food allergy is increasingly being recognized and investigated. It has been noted that individuals with food allergies may acquire different gut microbe composition compared to healthy control subjects [160]. Any disparities in the gut microbial diversity may contribute to food allergy development [161]. The effects of environment on allergic diseases were first implicated in the hygiene hypothesis. [162]. It is suggested that better sanitation and living conditions may reduce the exposure to pathogens but may increase the susceptibility to other allergic conditions. The infection in early childhood due to "unhygienic contact" with older siblings or through mothers prenatally may protect children against the development of hay fever. Also, family size has been associated with allergy susceptibility, as reduced family size and minimal exposure could increase atopic disease development [163,164]. The classical food allergy mechanism is characterized by the generation of an immune response against antigen through IgE antibodies [165]. The pre-immune Ig repertoire undergoes recombination to result in IgM production that aids

as the B cell receptor (BCR) [165,166]. When B cells are maturing, they undergo receptor editing, leading to changes in BCR specificity and facilitating B-cell tolerance development [165,166]. The commensal bacteria of the gut must generate an environment favouring B-cell class switch recombination to IgA than to IgE. The gut dysbiosis might allow increased allergen access to peripheral lymphoid tissue where cognate B cells undergo class switch recombination to IgE [166,167].

Many recent studies have commented upon commensal bacteria's role in regulating effector cells at sites of allergic inflammation [168]. In a model based on allergic airway inflammation, an increase in basophil numbers was reported in the airways of germ-free mice compared with SPF mice. In fact, germ-free mice revealed increased airway hyper-responsiveness following intranasal ovalbumin challenge compared to their SPF counterparts [168]. The exposure of antibiotics in mice that affects the commensal population of mice gut showed higher resting serum IgE levels and an increased frequency of circulating basophils [167,169]. Such studies provided a concept of commensal bacteria–IgE–basophil axis as there exist a correlation between an impaired commensal microbial signalling and elevated serum IgE due to increase in numbers of circulating basophils [169]. Identifying protective bacterial taxa, and their metabolic products such as short-chain fatty acids could provide an insight into their role in both allergic diseases such as asthma and food allergy. Hence, recent interest in the therapeutic that could modify or modulate the microbiome for treating allergic diseases in the form of biologically active natural compounds is now being developed.

1.13.1 Gut microbiome: A meditator of food allergy

The human intestine has various types of bacteria that adapt to the host's intestine environment, creating the intestinal microbiota. Diet and breast milk play a significant role during infancy as they influence the bacterial microbiota in humans in the early stages of life. Any dysbiosis in microbiota may contribute to the development of allergy [160]. The gut microbiota is in a constant state of flux dictated by our diet and lifestyle. Natural compounds can be used as a source to improve the commensal bacterial population to alleviate the dysbiosis of microbiota in diseased conditions such as food allergy.

The human gut typically harbours a diverse array of intestinal microbiota, which is dominated by four bacterial phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* [170]. Food proteins are hydrolysed through biochemical processes involving stomach acid, bile duct juices, and pancreatin and pepsin enzymes during the digestion process. These hydrolysing processes result in varying sizes of food proteins being broken down into smaller peptides while some remain intact. These peptides and intact proteins are subsequently presented to gut-associated lymphoid tissue (GALT) [161,171]. Early-life exposure to smaller peptides from hydrolysed proteins in the gut tends to aid oral tolerance induction under the right conditions [172]. GALT continuously distinguishes non-allergenic food antigens from allergenic food antigens to establish oral tolerance to specific food antigens [161,171]. Commensal bacteria in the gut can help this immune tolerance against food allergens by interacting with the gut's mucosal immune system (GALT) [166]. Immune tolerance seems to be primarily achieved during the early stages of life when the mucosal barrier and immune system are immature [173]. Early experiments using germ-free mice demonstrated that impaired function of $CD4^+CD25^+Foxp3^+$ Treg cells within the mesenteric lymph nodes and Peyer's patches could be key mediators in disrupting the oral tolerance [174–176]. Similarly, another study in germ-free mice demonstrated that early introduction of *Bacteroides fragilis* could reinstate the impaired GALT function and induce tolerance during the neonatal period by generating Treg cells via IL-10 instigated pathways [177]. Several studies have indicated that alterations in the diversity of commensal gut microbiota (dysbiosis) could lead to the development of food allergies or other diseases [166,173,178,179].

Still, the recent advances in treatment strategies are not satisfactory, and allergen avoidance is the only existing practical way. The use of natural supplements like green algae and various microbiome-based strategies in the field of food allergy can set the platform for the improved diagnostics, preventative, and treatment measures.

1.14 Knowledge gap in food allergy treatment research

Current research into natural products and probiotics has demonstrated potential beneficial effects on the human immune system and gut, with promising results in the prevention and treatment of various diseases. The current literature also shows positive effects of these natural sources in suppressing allergic symptoms, primarily through

reducing the generation of allergen-specific IgE antibodies, downregulation of effector cell activation (e.g., mast cells) or expression of Th2 cytokines assisting in the progression of allergic reactions.

Screening for an alternate therapeutic approach is an important avenue, and preclinical research serves as a crucial stepping-stone in identifying potential therapeutic candidates for human use. It is essential that efficacy is investigated in a streamlined manner (e.g., *in vitro*, *in vivo* and phase 1 and 2 clinical trials) to reaffirm the safety and usefulness of such compounds. In general, mouse models that are currently being used for inducing food allergy have some intrinsic limitations. Presently, mouse models of food allergy are developed with the help of adjuvants (e.g., alum, Freund's complete adjuvant and cholera toxin B) in genetically distinct mouse strains (e.g. BALB/c or C3H/HeOuJ) using different routes of sensitization (e.g. intraperitoneal and intragastric) [79,180,181]. Hence, most studies reporting the efficacy in murine systems tend to fail or show no effectiveness when replicated in clinical studies [182]. Proper reporting of studies by following the ARRIVE guidelines set for reporting research conducted in animals can help overcome some of these issues plaguing the reproducibility and translation in preclinical research [183].

Allergen-specific immunotherapy is primarily based on the whole extract, purified proteins or peptides and designed to be very specific to the implicated food allergen source, e.g., peanut allergens. However, natural product-based approaches appear to reduce the symptoms and/or target specific pathways independent of the implicated food source. This is a major advantage of such type of approaches for two reasons, a) a single therapeutic approach can target several different types of food allergens, a medical state which is observed in most allergy sufferers with multiple co-sensitization and allergy to different types of foods, and b) the cost of research and development for such broad range therapeutics is much lower as compared to AIT, which must be established and validated for every single source of an allergenic food.

Such innovative strategies combining allergen-specific immunotherapy with natural bioactive compounds into a suitable dosage regimen may potentially have a safe and effective treatment strategy for food allergies.

1.15 Summary and synopsis of the thesis

The research work presented in this PhD thesis is composed under separate chapters as follows: the current chapter summarises the mechanism of food allergy and natural products' role as a rich source of biologically relevant compounds and their functional aspect in modulating food allergy in different biological models. The research highlighted in this chapter also outlines the current status and prevalence of peanut allergy worldwide and the role of tree nut allergens as potential cross-reactive protein sources.

Chapter 2 discusses the effect of feeding the crude extract and isolated purified polysaccharide from green macroalgae *Ulva ohnoi* (*U. ohnoi*) in a mouse model of peanut allergy. As a preliminary analysis, we also studied the toxicity of crude extract of *U. ohnoi* and measured the effect of feeding on various biochemical parameters.

Chapter 3 investigated the impact of the two separate housing facilities (conventional and specific-pathogen-free [SPF]) on the clinical and immunological parameters in a peanut allergy mouse model, based on the challenges faced in developing the model in Chapter 2. Changes in the microbiome composition of mice housed in two different facilities were investigated.

Chapter 4 details the effect of feeding the algal polysaccharide and carotenoid on the mouse gut microbiome, to investigate whether the changes in gut microbial diversity may skew towards tolerance or allergic sensitization. The impact of purified ulvan and astaxanthin feeding on intestinal microbiota in mice was evaluated using 16s bacterial microbiome sequencing in collected faeces samples.

Chapter 5 discusses the IgE reactivity in nut sensitized patient serum to macadamia nuts and its purified proteins. Cross-IgE binding between macadamia nuts and purified proteins was assayed utilizing the grid-blot immunoassay and ELISA.

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Chapter-2

Safety and efficacy of marine algal compounds

Publication in progress:

Safety and immunological efficacy of bioactive compounds from marine algae *Ulva ohnoi* in a mouse model of peanut allergy.

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2.1 Introduction

Peanut is consumed worldwide in various forms such as snack, oil, flour, and a food source with significant nutritional value. On the contrary, peanut is also the most common food allergen source known to result in nearly 6-8% of allergy incidences in children[1–3]. Further reports suggest that incidences are projected to rise in the coming years[1]. As opposed to other food allergies, peanut allergy is often not outgrown in adulthood[4]. Peanut allergy is mediated by IgE antibody upon exposure and regulated by Th2 cytokines that mount the typical allergic response and, in severe cases, can lead to anaphylactic shock[5].

In recent years, researchers have shown interest in green macroalgae (Chlorophyta), especially from the genus *Ulva* which is edible and has been reported to possess nutritional and remedial benefits[6,7]. Ulvan is a cell wall polysaccharide composed of basic structural units including xylose, rhamnose and uronic acids and these subunits acts as fundamental units of Ulva[8]. The structural units of Ulva contribute to the overall dry weight biomass of the ulvan and act as bioactive components to modulate cellular signalling[6,9]. Consequently, considerable interest has grown for Ulva as natural products that can be utilized for human health interventions, industrial and agricultural purposes[8,10–13]. Various reports have also confirmed that green macroalgae can serve multiple purposes ranging from immune-modulating compounds with antioxidant, anticancer and anti-allergic properties and can also act as functional food sources[6–8,14,15]. Under the genus *Ulva*, a recently characterized seaweed *Ulva ohnoi (U. Ohnoi)* has been reported for possessing immunomodulatory properties[9].

Despite various studies reporting the immunomodulatory properties of ulvan, the effects of ulvan biomass or ulvan isolated from *U. ohnoi* to suppress allergic reactions remains understudied. A recent study examined the sub chronic toxicity of *Ulva pertusa* in rats and reported no toxicological effects even at 600 mg/kg bw[16]. In the current study, we report for the first time the toxicological safety assessment of *U. ohnoi* isolated biomass and ulvan compound administered in the BALB/c mice. Additionally, we also assessed the efficacy of crude ulvan biomass and purified ulvan isolated from *U. ohnoi* on a peanut-induced allergy model in mice. The toxicological assessment reveals that overall, there were no significant changes in the serum biochemistry of the mice

administered with a 5mg/day/mouse dose of crude biomass or purified ulvan. There seems to be some mild oxidative stress generation in the liver due to crude biomass feeding; however, superoxide dismutase enzyme activity measured in liver tissue isolates suggested the optimal inhibition mitigation of oxidative stress generated by crude biomass administration effectively. In the allergy model, we demonstrated that crude ulvan biomass exacerbates a peanut specific IgG1 response compared to purified ulvan. The development of a Th2 response is supported by the production of cytokines including, IL-4, IL-5 and IL-13 production, show an increase in mice administered with crude ulvan biomass compared to purified ulvan. In contrast, upon prolonged feeding of purified ulvan, peanut-specific IgG and IgG1 showed a decreasing trend indicating the suppression of the Th2 response over time. Although not significant, a decrease in Th2 cytokine (IL-4, 5 and 13) response was evident in mice treated with purified ulvan for a longer duration, supporting the immune suppressive activity.

2.2 Materials and methods

2.2.1 Animals

6-8 weeks old female BALB/c mice for toxicology (n=2-4 per group) and peanut allergy (n=6 per group) studies were obtained from the breeding facility of the Australian Institute of Tropical Health and Medicine (AITHM) at James Cook University, Townsville, Australia. The mice were maintained on an *ad libitum* water and specialized (nut or soy-free) diet (SF06-053, Speciality feeds, Western Australia) to avoid possible recognition of cross-reactive allergen by immune system. Small animal husbandry and care were carried out per the protocols and recommendations from an independent ethics committee for animal experimentation (the Animal Ethical Committee of James Cook University, Townsville, Australia, and Ethics ID: A2426).

2.2.2 Reagents

Peanut extract was prepared from human consumption quality peanut flour from Golden Peanut Speciality Products Division, Georgia, USA (www.goldenpeanut.com). Briefly, the peanut flour was mixed in 1× PBS and left overnight at 4°C on a shaker a day before use. Peanut extract for activating the splenocyte culture was prepared by collecting the

supernatant by centrifuging the peanut slurry at 10,000 rpm at 4°C. Cholera toxin (CT) and concanavalin A (Con A) was purchased from Sigma Aldrich (Melbourne, Australia). Crude ulvan biomass (CB) and purified ulvan (PU) were refined from *U. Ohnoi* and manufactured by Marinova Pty Ltd (Tasmania, Australia) using a proprietary aqueous extraction process[17]. Serum biochemistry on toxicology group samples was performed using the alanine transaminase (ALT) (ab105134), aspartate aminotransferase (AST) (ab105135) and lactate dehydrogenase (LDH) (ab102526) assay (Abcam, Victoria, Australia). The oxidative stress markers superoxide dismutase (SOD) (ab65354), and lipid peroxidation (MDA) (ab118970) were measured activity assay kits (Abcam, Victoria, Australia). Mouse IgG, IgG1 and IgG2a antibody used for detecting peanut-specific antibody titre were obtained from Life Technologies (Mulgrave, Victoria, Australia). Coating buffer for ELISA was prepared using sodium carbonate (0.2M) and sodium bicarbonate (0.2M) with pH-9.6 using double distilled water. Washing buffer (PBS-tween) solution for ELISA was prepared by mixing 1X PBS with 0.05% tween solution. Blocking solution for ELISA was prepared using a 1:9 ratio of 10X casein solution (company, country) in PBS-tween solution. Antibody buffer for ELISA was prepared using the .05X casein solution. TMB solution (Life Technologies, Melbourne, Australia) was used to develop the ELISA reaction. MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) kit was used to detect IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-13, and IL-17 in stimulated splenocyte, was purchased from Merck Millipore (Melbourne, Australia), and levels were analysed using the MAGPIX system (Merck, USA).

2.2.3 Estimation of protein concentration

Peanut extract was prepared to activate the splenocytes isolated from the allergic and ulvan administered mice. Protein estimation was performed as instructed in the manual provided with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Briefly, 10 µl of Bovine serum albumin (BSA) standards were diluted (1:2) in PBS in different microcentrifuge tubes. PBS buffer was used as the blank standard, and the provided 2 mg/ml standard was used as the highest concentration standard. Solution A and B provided in the kit were mixed well and diluted 50:1 for the final reaction. 10 µl of neat and diluted peanut extract samples were prepared for the estimation. The reaction was set up by adding 200 µl of Solution A and B mixture in each standard and sample tube

and incubated at 50-55 °C for 15 min in a water bath. After the incubation, the tubes were centrifuged briefly, and 100 µl of reaction from each tube was transferred into 96-well plates. The plate with the reaction volumes was cooled at room temperature. Subsequently, the plate was read at 562 nm using a spectrophotometer. The 4-parameter logistic curve was generated, and blank corrected readings were used to calculate the final concentrations in mg/ml.

2.2.4 Assessment of toxicity in polysaccharide fed mice

A pilot study was conducted to evaluate the toxicological effects of CB and PU feeding on mice at a lower dose based on previously reported studies. Due to the project timelines and commitments, dose-curve response studies could not be conducted to study the cytotoxicity and toxicity of crude ulvan biomass and purified ulvan. Therefore, the pilot trial was performed in accordance with ethics approval with minimum use of mice for toxicity study. The experimental animals were divided into three different groups (n = 2-4). Group 1 served as a naïve group (n=2) that received PBS via oral gavage and *ad libitum* water and feed for 4-weeks [Fig 2.1]. Group 2 and 3 mice served as crude biomass fed (CB) (n=4) and purified ulvan fed (PU) mice receiving 5mg CB or PU/mouse in PBS via oral gavage daily with *ad libitum* drinking water and feed for 4-weeks [Fig 2.1]. Mice were monitored for any physical discomfort and visible clinical signs. Bodyweight was measured for groups every consecutive week. Mice were euthanized after 4-weeks, and blood was collected for serum isolation to assess the serum biochemistry. Mice livers were harvested and stored at -80°C for measuring the biochemical and oxidative stress parameters.

2.2.5 Estimation of serum biochemical composition

Blood from naïve, CB and PU groups of mice was collected while dissecting using heart puncture. The blood was clotted at room temperature and subsequently centrifuged at 10000 rpm for 5 min. at 4ºC. Serum was collected for estimating the levels of biochemical and oxidative stress parameters, alanine transaminase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), Lactate dehydrogenase (LDH) and lipid peroxidation

(MDA). The levels were quantitated using a commercially available assay kit according to the manufacturer's protocols that are explained briefly below.

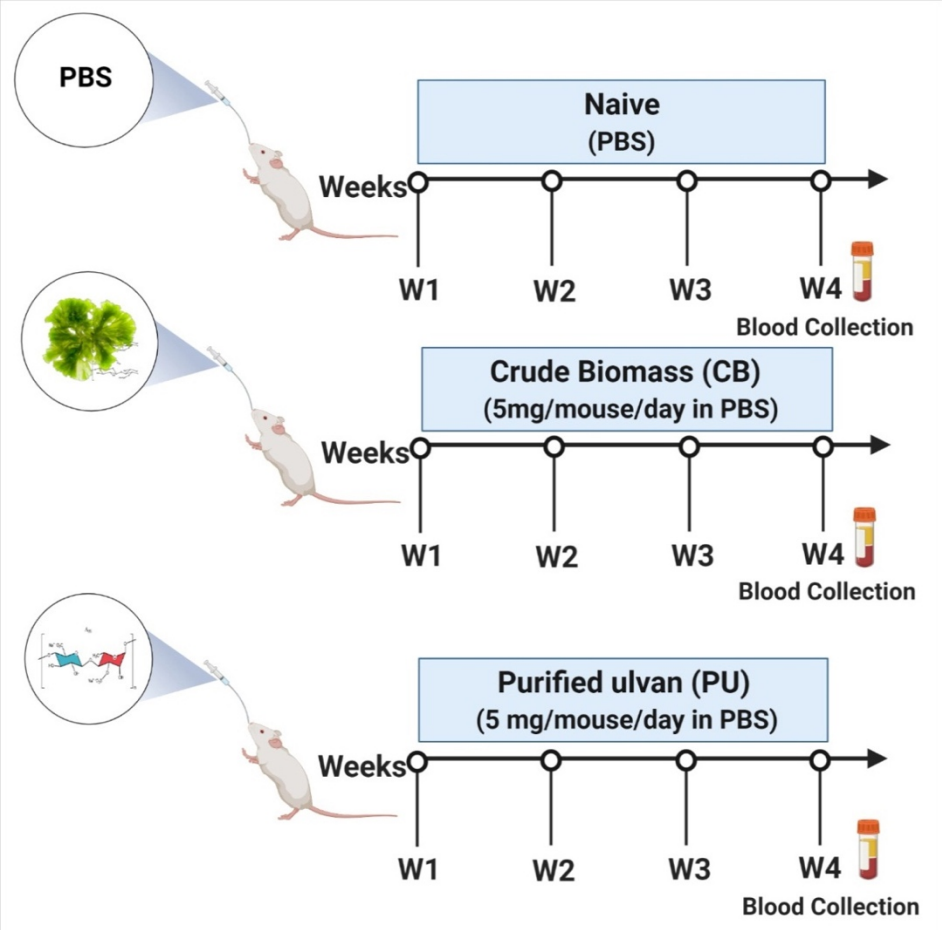

Fig 2.1: Toxicity assessment of crude biomass (CB) and purified ulvan (PU) in BALB/c mice. Timeline depicting the feeding regimen of CB and PU at a specified dose of 5 mg/mouse/day administered intragastrically by dissolving in 200 µl PBS. Mice were fed every day for four weeks, with naïve mice receiving only PBS. Mice were monitored for any physical discomfort, and body weight was measured for four weeks. Mice were sacrificed after four weeks; blood and liver tissue samples were collected for toxicological assessment.

2.2.6 Alanine transaminase (ALT) assay

The ALT assay was performed to estimate the alanine transaminase enzyme activity on pyruvate due to the polysaccharide feeding in mice. The protocol was followed as instructed; a standard curve (0-10 nmol/well) was prepared using the standards provided. Serum samples and kit contents were brought to room temperature, and a reaction mix was prepared. The plate was designed for duplicated replicates by adding 20 µl of standard solution, diluted serum samples (1:100) and positive controls into the

wells. The enzyme reaction mixture (100 µl) was prepared and added to all the sample containing wells. The microplate was incubated for initial 10 mins and read in a kinetic mode at 570 nm for 60 mins with readings recorded every 10 min as instructed. ALT enzyme activity is calculated by measuring the pyruvate concentration in the sample well. Following calculations were performed for the estimation:

$$\text{ALT activity} = \left(\frac{B}{\Delta T x V}\right) \times D$$

B = amount of pyruvate (nmol) from standard curve, ΔT = reaction time (mins), V = volume used of original sample (in mL). D = sample dilution factor, units = mU/mL

2.2.7 Aspartate aminotransferase (AST) assay

The AST assay was performed to estimate the aspartate aminotransferase enzyme activity on glutamate because of the polysaccharide feeding in mice. The protocol was very similar to the ALT estimation activity assay, where a standard curve (0-10 nmol/well) was prepared using the standards provided within the kit. The assay preparations were performed at room temperature. The microplate was sampled in duplicate by adding 20 µl of standard solution, diluted serum samples (1:100) and positive controls into the wells. The enzyme reaction mixture (100 µl) was prepared and added to all the sample containing wells. The microplate was incubated for initial 10 mins and read in a kinetic mode at 450 nm for 60 mins at an interval of 10 min as instructed. AST enzyme activity is calculated by measuring the glutamate concentration in the sample wells. Following calculations were performed for the estimation:

$$\text{AST activity} = \frac{B}{(T_2 - T_1) \times v} \times D$$

B = amount of glutamate (nmol) from standard curve, T_2 - T_1 = reaction time (mins), V = volume used of original sample (in mL). D = sample dilution factor, units = mU/mL

2.2.8 Lactate dehydrogenase (LDH) assay

The LDH assay was performed to estimate the enzyme lactate dehydrogenase activity, an oxidoreductase enzyme, to determine the effect of polysaccharide feeding on mice liver health. In brief, a standard curve (0-12.5 nmol/well) was generated using the

standards provided in the kit. The assay was performed at room temperature, and all the kit materials were kept under dark before use. The microplate was prepared by adding 50 µl of standard solution, diluted serum samples and positive controls into the designated wells in duplicates. The enzyme reaction mixture (50 µl) was prepared and added to all the sample containing wells. The microplate was immediately read every 2-3 minutes at 450 nm for at least 60 mins at 37º C under dark conditions. LDH enzyme activity is calculated by measuring the amount of enzyme that acts as a catalyst for converting lactate to pyruvate to generate NADH/ min of the reaction. Following calculations were performed for the estimation:

$$\text{LDH activity} = \left(\frac{\text{B}}{\Delta\text{TxV}}\right) \times \text{D}$$

B = amount of NADH (nmol) from standard curve, ΔT = reaction time (mins), V = volume used of original sample (in mL). D = sample dilution factor, units = mU/mL

2.2.9 Superoxide dismutase enzyme activity assay

The SOD activity assay was performed to determine the antioxidative activity of the enzyme to inhibit xanthine oxide radicals. In brief, kit materials and samples were brought to room temperature for the assay to estimate the optimum enzyme activity. Supernatant from liver tissue samples (10 mg) was isolated as per the instruction provided in the kit. In the microplate, 20 ul of each Blank-1 (ddH_2O), Blank-2 (pooled sample), Blank-3 (ddH_2O) and liver tissue supernatant samples were added to the wells. Enzyme working solutions were added using multi-channel pipettes as instructed in the kit protocol scheme. The sample and enzyme solutions were appropriately mixed and incubated at 37ºC for 20 mins. After the incubation, the microplate was read for measuring the OD at 450 nm. Following calculations were performed for estimating the SOD enzyme activity:

$$\text{SOD activity (inhibition rate \%)} = \frac{[(\text{Ablank1} - \text{Ablank3}) - (\text{Asample} - \text{Ablank2})]}{\text{Ablank1} - \text{Ablank3}} \times 100$$

A -= absorbance, units: % inhibition

2.2.10 Lipid peroxidation (MDA) assay

A lipid peroxidation assay was performed to determine the oxidative damage that may have happened after polysaccharide feeding to mice. Malondialdehyde (MDA) concentrations are measured to determine the lipid peroxidation activity where oxidative degradation of lipids happens locally or systemically. In brief, a standard curve (0-20 µM) was generated using the MDA standards provided in the kit. The assay kit was equilibrated at room temperature, and solutions for the assay were prepared as instructed. Liver tissue (10 mg) was homogenized, and the supernatant was isolated as recommended in the protocol. The microplate was prepared by adding 200 µl of standard solution and diluted serum samples in designated wells. The TBA reagent provided in the kit was added to all previously sampled wells and incubated at 95º C for 60 minutes. The microplate was cooled for 10 mins, and 200 µl of MDA-TBA adduct was aspirated into a separate plate for analysis. The microplate was immediately read at 532 nm. The concentration of MDA in the samples was calculated using the following formula:

$$\text{MDA concentration} = \left(\frac{\text{A}}{[\text{mg}]}\right) \times 4 \times \text{D}$$

A = amount of MDA (nmol) from standard curve, mg = original tissue amount used, 4 = correction for using 200 µl of the 800 µl reaction mix, D = sample dilution factor, units = nmol/mL

2.2.11 Induction of peanut allergy by oral gavage of peanut extract

Female 6–8-week-old mice were grouped into five groups, naïve, peanut allergy (PA) and three treatment groups (T1-T3). Briefly, PA mice were sensitized using intragastric (IG) routes from week 1 to week 5 with 10 mg PS, 20 µg CT and 10% sodium bicarbonate mix doses [Fig.2.2]. Intragastrical challenges (200 mg/mouse) were performed in two phases, starting from week 6 to week 8 and week 10 to week 12 with two-week gap [Fig.2.2]. Symptomatic checks were performed for 30-60 min after each challenge. Blood samples were collected post sensitization and challenge at different intervals for measuring the antibody levels in serum [Fig. 2.2]. In addition, spleens were

collected in RPMI 1640 medium containing 10% fetal bovine serum (FBS) for splenocyte culturing.

2.2.12 Polysaccharide feeding to peanut-allergic mice

The treatment groups were categorized based on the starting week of the polysaccharide feeding in peanut-allergic mice. Crude ulvan biomass (CB) and purified ulvan (PU) feeding was performed in three different regimens: T-1 (W8-W10), T-2 (W10-W12), and T-3 (W8-W12) [Fig 2.2]. Mice were fed with CB and PU with a fixed dose of 5 mg in 200µl of PBS on alternate days in three different regimens.

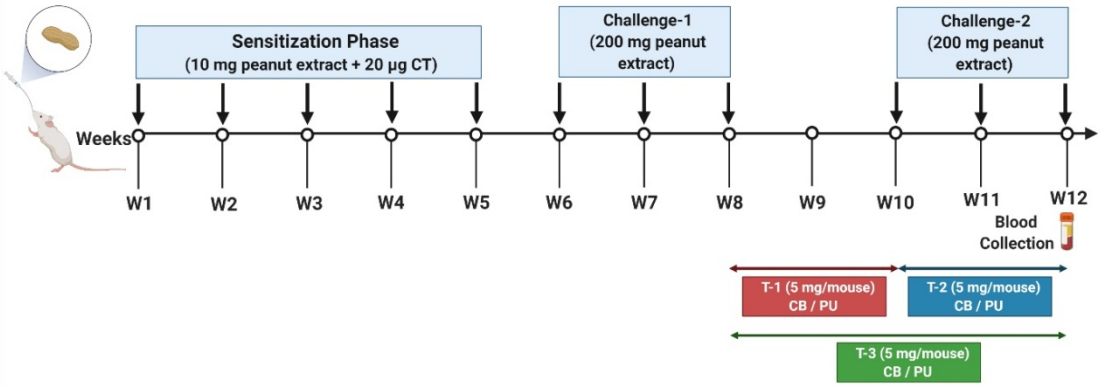

Fig 2.2: Effects of CB and PU administration on allergic symptoms in peanut-sensitised mice. Timeline depicting the protocol for the induction of peanut allergy in a mouse model. Groups of mice (n = 6 mice) were sensitised with peanut (10 mg/mouse) and cholera toxin (20 µg/mouse) by oral administration on consecutive weeks (W1-W5). Peanut-sensitised mice were orally challenged in two terms, challenge-1 (W6-8) and challenge-2 (W10-12), with a one-week gap (W9). Mice were orally treated with CB and PU (5mg/mouse) in three terms, T-1 (W8-W10), T-2 (W10-W12) and T-3 (W8-W12) every other day. Mice were sacrificed after W12; blood and tissue samples were collected and stored for further examination.

2.2.13 Serum antibody and cultured supernatant cytokine level measurement

2.2.13.1 Peanut-specific serum antibody response measurement

Blood samples for serum isolation were collected at different time intervals during sensitization and challenge phases, as represented in Fig 2.2. ELISA was performed to determine the peanut-specific IgG, IgG1, and IgG2a levels in serum. Briefly, 96 well plates were coated using the peanut extract (10 µg/ml of coating buffer) as an antigen to appropriate wells. The plate was incubated overnight at 2-8 ºC. The following day, 250 µl of blocking solution was added to the appropriate wells, and the plate incubated at room

temperature for 1 hr. After the incubation, the plate was washed once, and diluted serum samples (1:100) were added to appropriate wells, and the plate again incubated for 2 hrs at room temperature. After incubation, the wells were washed three times using PBS-0.05% tween solution. Subsequently, 100 µl of secondary rat anti-mouse antibody (IgG, IgG1 and IgG2a) with 1:1000 dilution was added to the appropriate wells. The plate was incubated at room temperature for 1 hr and washed five times using the PBS-tween solution. Residual wash buffer in the wells was tapped out gently on an absorbent paper towel. Next, 100 µl of streptavidin-conjugated anti-mouse IgG antibody with 1:10000 dilution was added to appropriate wells, and the plate was incubated for 30 mins at room temperature. The plates were later washed five times, and residual fluid was removed by tapping on a paper towel. ELISA plate was developed using 50 µl of TMB solution (1:1 solution A and solution B) added to appropriate wells, and the plate was kept in the dark for 10-15 min (Based on colour development). Next, 50 µl/well of 1M HCL solution was added to all the wells to stop the ELISA reaction, and absorbance reading was measured at 450 nm. Antibody levels were reported as optical density (OD) measured at 450 nm.

2.2.14 Stimulation of splenocyte using peanut extract

Spleens from all groups were collected aseptically, and single-cell suspensions of splenocytes were prepared. Briefly, spleens were homogenized in RPMI-1640 containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution, and single-cell suspensions were collected using the cell strainer. The cell suspension was treated with red blood cell lysing buffer. Treated cells (2×10^5/well) were supplemented with the same base media preparation of RPMI-1640 and cultured in a flat-bottom 96-well plate (Nunc) in the absence or the presence of peanut extract (100 µg/well) and with con A (2.5 µg/mL/well). The plates were incubated for 72 h at 37°C with 5% CO_2. The supernatant was collected after 72 hrs and stored in -80°C for further analysis.

2.2.15 Cytokine measurement using MAGPIX multiplex assay

To measure cytokine levels in the supernatant of peanut stimulated splenocyte, an MAGPIX multiplex assay was performed using the manufacturer's instruction. Briefly, the assay plate was washed for 10 mins on a plate shaker at room temperature by adding

200µl of wash buffer into each well of the plate. Wash buffer was decanted, and residual fluid tapped out from the plate on an absorbent paper towel. 25 µl of each standard or quality control were added into the appropriate wells. Assay buffer was used as a background as 0 pg/mL standard. Subsequently, 25µl of assay buffer was added to the sample wells as per controls and experimental groups. RPMI-1640 culture medium was then added as a matrix solution to the appropriate wells, such as backgrounds, standards, and control wells. The supernatant (25µl) was later added into the appropriate wells. The pre-mixed bead solution provided in the kit was vortexed for 10 sec and added into the appropriate wells containing background, controls, standards, and samples. The plate was sealed and stored at 4°C on an orbital shaker overnight. The plate was processed the following day by gently removing the well's content and washed twice as instructed in the manual. After washing, the plates were gently tapped to remove residual fluid, and 25 µl of detection antibodies were added to individual wells and incubated for 1hr at room temperature on a plate shaker. After incubation, 25 µl streptavidin-phycoerythrin solution to individuals wells. The plate was sealed using foil wrap and incubated for 30 mins on a plate shaker at room temperature. After incubation, plate content was gently removed, and the plate was washed two times as instructed. For running the assay 150 µl drive fluid was added to the wells. The plate was incubated on a shaker momentarily for 5 mins to resuspend the beads. Plate with beads solution was then run and analysed using the MAGPIX with xPONENT software. The Median Fluorescent Intensity (MFI) data was computed using a 5-parameter logistic curve-fitting method by the proprietary software for calculating cytokine/chemokines concentrations in samples.

2.2.16 Statistical analysis

The differences in the biochemistry profiles, oxidative stress markers and peanut-specific antibody levels between treated groups were compared using one-way ANOVA with Dunnett's multiple comparison test. In addition, the Kruskal-Wallis test was performed to compare the difference between cytokine levels between appropriate mouse groups using Prism 9.02. Data are presented as mean ± standard deviation (SD) of the observed values. *p* values computed to be less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Estimation of toxicological effects of green algae products in mice

Various studies have reported on the efficacy of bioactive compounds from green algae; however, information on toxicological assessment is scarce. In the present study, we assessed the toxicity of CB and PU isolated from green algae *U. ohnoi*. Mice were fed with 5mg of CB and PU dissolved in PBS for four weeks and monitored for their weight change and adverse clinical symptoms. Assessment of any adverse physiological effects of CB and PU feeding was conducted using the serum biochemistry (ALT, AST, LDH) and oxidative stress markers (MDA and SOD) in hepatic tissue.

2.3.2 Clinical and weight change assessment

Mice in groups CB and PU were monitored for 15-30 mins after the feeding regimen and daily for any late adverse physiological reaction. Mice body weight increased over four weeks in all groups. Bodyweight increase in naïve group and PU group was almost similar; however, CB group weight change was significantly lower at week three compared to the naïve group [Fig 2.3].

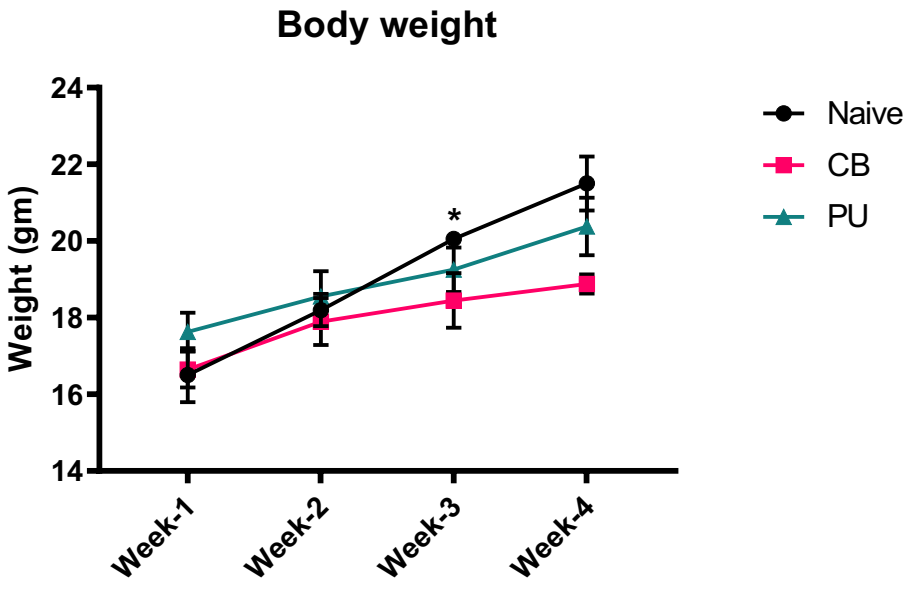

Fig 2.3: Bodyweight assessment of mice fed with crude biomass (CB) and purified Ulvan (PU) for four weeks. Two-way ANOVA was performed, and data represent the mean ± SD (n=2-4). *$p < 0.05$.

2.3.3 Green algae treated mice maintained the levels of ALT, AST and LDH

Serum biochemistry analysis is an important marker for analysing the elevated serum enzymes levels such as ALT, AST and LDH that are the markers of hepatic damage. ALT is a critical enzyme reported for catalysing the alanine cycle and indicative of any hepatic damage. In the present study, we measured the ALT enzyme in serum isolated from CB and PU mice. An increasing trend in ALT enzyme was observed in PU mice and more so in CB group compared to naïve following 4 weeks of dosing; however, no significant differences were present [Fig 2.4a]. AST enzyme is an indicator of amino acid metabolism in hepatic tissue. AST enzyme levels in the CB treated group were significantly higher as compared to the naïve group. However, no significant difference was noted in the PU group [Fig 2.4b].

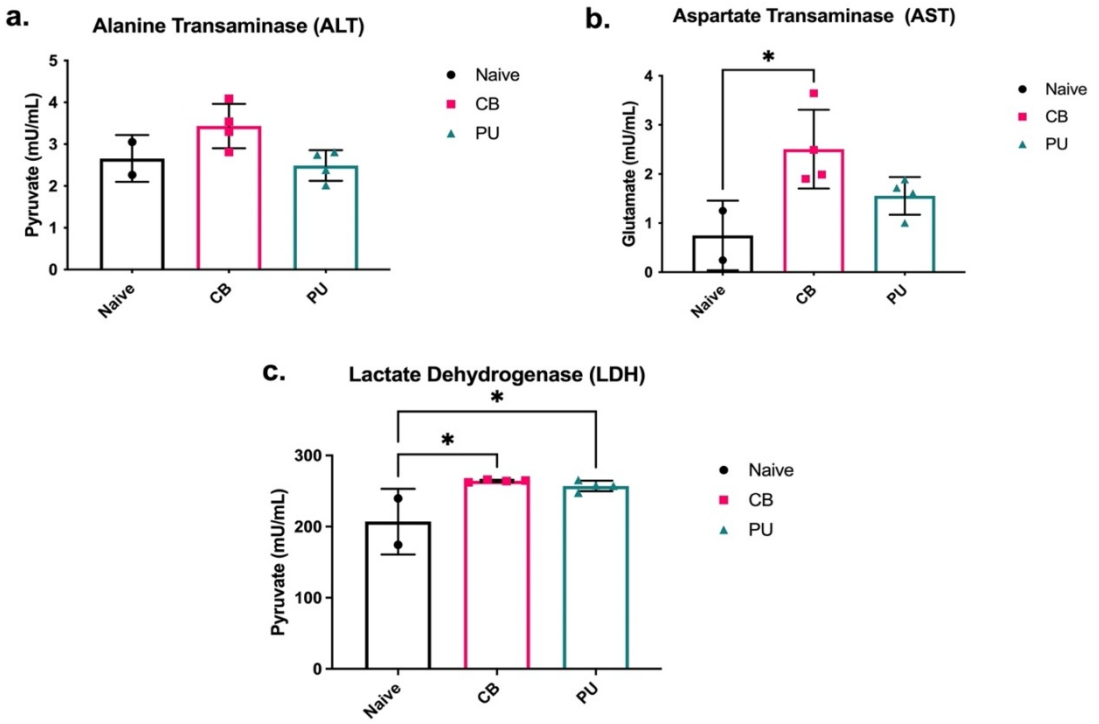

Fig 2.4: Effect of feeding crude biomass (CB) and purified ulvan (PU) from *U. ohnoi* on serum enzyme reactivity in BALB/c mice. ALT: alanine transaminases; AST: aspartate transaminase: LDH: lactate dehydrogenase. One-way ANOVA was performed, and data represents the mean ± SD (n=2-4) *$p < 0.05$ significant as compared to naïve.

An increasing trend in ALT enzyme levels and a significant increase in AST in CB group could indicate oxidative stress in hepatic tissue and hence the slow rate in weight gain as

observed [Fig 2.3]. LDH is an oxidoreductase enzyme that is released in the blood in case of tissue injury. LDH levels were observed to be significantly higher in both CB and PU administered groups as compared to naïve, further indicating the presence of mild oxidative stress response generation upon administration of CB and PU [Fig 2.4c].

2.3.4 Green algae administration improves antioxidative parameters

Ulvans from green algae has been reported for their antioxidative properties[6,7,15]. To confirm these findings the Liver from CB and PU treated mice were excised, and the oxidative stress marker (LPO) and antioxidant (SOD) markers measured. Lipid peroxidation of polyunsaturated fatty acids (PUFA) could produce MDA indicating cellular damage because of oxidative stress. As evident from the serum biochemical parameters, CB administration could induce mild oxidative stress. In the present study, MDA levels in the supernatant isolated from hepatic tissues were measured using an LPO assay. MDA levels in naïve and CB groups were similar, whereas lower levels of MDA were observed in the PU group [Fig 2.5a]. However, no significant differences were present across all groups. Another critical factor in oxidative stress condition is the formation of free radicals. Free radical generation could likely cause tissue damage and induce oxidative stress. Superoxide Dismutase (SOD) is an essential antioxidative enzyme that can be measured to analyse xanthine oxide free radical inhibition in a SOD enzyme activity assay.

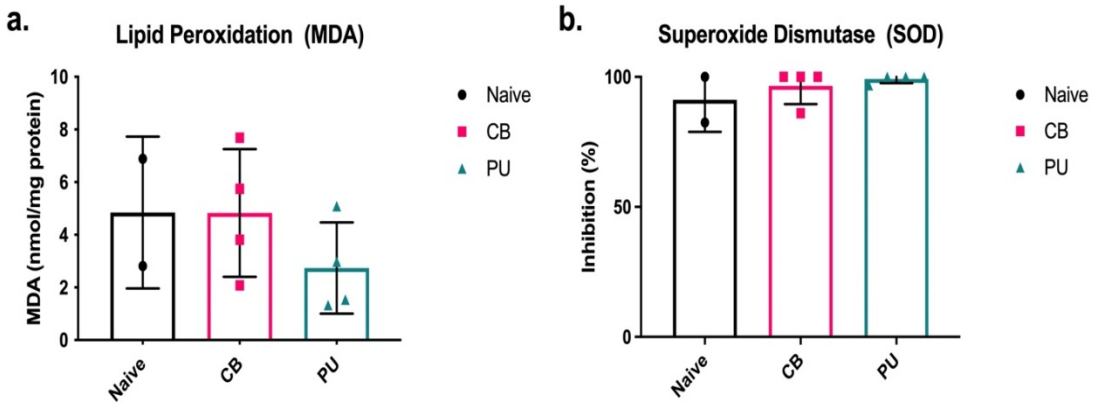

Fig 2.5: Effect of feeding crude biomass (CB) and purified ulvan (PU) from *U. ohnoi* on oxidative stress parameters measured in liver tissue isolates from BALB/c mice. LPO, lipid peroxidation; SOD, superoxide dismutase. One-way ANOVA was performed, and data represents the mean ± SD (n=2-4) *$p < 0.05$ significant as compared to naïve.

Interestingly, CB and PU group showed higher percent inhibition (%) as compared to naïve [Fig 2.5b]. These results confirm that mild oxidative stress was generated upon CB and PU administration, and SOD activity was optimal in mitigating the effects. Overall, no significant cellular toxicity or oxidative stress was observed in CB and PU (5 mg) administered mice after four weeks of feeding.

2.3.5 Murine model of peanut-allergy and impact of green algae products

To determine the effects of the oral administration of CB and PU in peanut-allergic mice, mice were fed with CB and PU in three different regimens starting from week-8 after the first challenge phase. Following challenge, mice were observed for allergic symptoms such as diarrhea, abnormal posture, hunchback, reduced mobility, and lack of grooming were noted in allergic mice, and each individual mouse was monitored over a period of 30–60 min after the oral challenge. The scoring of allergic symptoms ranged from 0-3 (0-No symptomatic change, 1-change in comfort levels (decreased mobility and grooming frequency), 2- Mild discomfort, intermittent diarrheal episodes, and less movement, 3-extreme diarrhea, loss of movement and postural changes). All groups, including peanut-allergic, CB treated, and PU treated mice, exhibited no extreme allergic symptoms apart from the slight discomfort after challenging with a high dose of peanut extract. (Observation data not shown).

2.3.6 Crude ulvan biomass and purified ulvan alter serum peanut-specific antibodies titres

To determine the effects of oral dosing of CB and PU on B-cell reactivity, the generation of peanut-specific immunoglobulin titres, IgG, IgG1 and IgG2a, were quantified. Allergen-specific immunoglobulins indicate an established immunological reactivity against the allergen source used for induction of an allergic response. The prolonged administration of CB and PU, as shown in the T-3 regimen, moderately suppressed allergen-specific titres [Fig 2.6]. However, CB administration does seem to exacerbate peanut-specific IgG levels [Fig 2.6a]. In particular, a significant increase in peanut-specific IgG1 antibody levels suggests that CB administration assisted in the build-up of immunological reactivity against peanut allergens in the T-1 and T-2 group, whereas not significant. However, a drop in the levels was observed in the T-3 group

otherwise [Fig 2.6c]. In addition, peanut specific IgG2a levels remain comparable across all groups, which could indicate there was no overall suppression of immunological reactivity against peanut upon CB administration [Fig 2.6e].

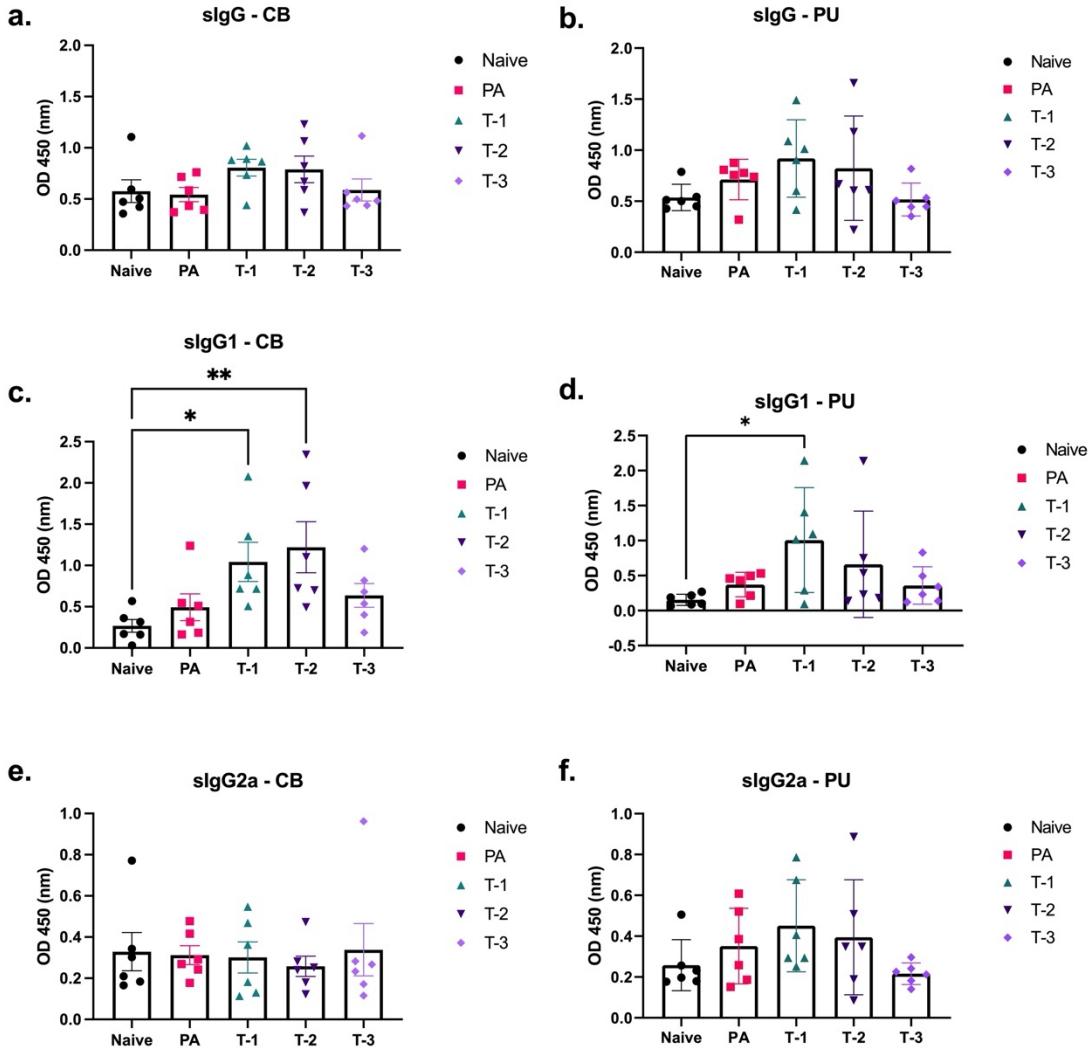

Fig 2.6: Peanut specific serum sIgG, sIgG1, and sIgG2a levels, as measured by ELISA. PA groups were sensitised from W1-W5 and later challenged from W6-W8 and W9-W12. Blood was collected on W12. Data are represented as mean ± SD of OD values measured at different weeks with n = 6 mice/group. Statistical analysis was performed using a one-way ANOVA, and Dunnett's test for multiple comparisons was performed. *$p < 0.05$, ** $p < 0.001$, *PA, peanut allergy, CB, crude ulvan biomass, PU, purified ulvan, T-1, T-2 and T-3, three different regimens were based on the administration period's length.*

In contrast, PU administration overall does increase the total peanut-specific IgG titres [Fig 2.6b]. However, a clear decreasing trend in peanut-specific IgG1 antibody level was observed in T-2 and T-3 regimen compared to T-1 regimen [Fig 2.6d]. Interestingly,

although the peanut-specific IgG1 titres were elevated in the T-1 and T-2 regimen compared to the peanut allergy group, an increasing trend in IgG2a levels was also observed in both treatment groups. [Fig 2.6d, f]. Although not significant but elevated levels of IgG2a levels suggest that systemic suppression of immunological reactivity towards peanut allergens was mildly mitigated upon PU administration [Fig 2.6d, f]. These results demonstrate that overall CB administration in peanut-allergic mice may have exacerbated peanut allergens' response, whereas PU administration indicate suppressive tendencies. Thus, current data suggest that PU could act as an immunomodulatory compound; however, further extensive investigation is required using a robust model.

2.3.7 Purified ulvan suppressed Th2 cytokines produced by stimulated splenocytes

The allergic response to peanut is primarily a Th2 skewed immune response, and to determine whether the orally administered CB and PU affected the systemic Th1/Th2 balance, cytokines levels in stimulated splenocytes supernatant were measured. The primary mediators of a Th2 response are IL-4 and IL-5, which are critical for activating and localization of eosinophils, whereas IL-13 is involved in mounting an inflammatory reaction[14]. In the CB administered group, a significant increase in IL-4, IL-5 and IL-13 was observed, even more than the peanut allergy group (PA) [Fig 2.7a, b and c]. Thus, increased Th2 cytokines in the CB administered group could indicate that a cellular response was generated, and CB administration further exacerbated the Th2 cytokine response. Furthermore, no significant differences were observed in IL-2, IFN-γ, IL-17 and TNF-α levels in the CB administered group. However, an increasing trend for IFN-γ was observed in the T-3 group, which had the most prolonged PU administration interval.

In contrast, although the PU administered groups demonstrated no significant changes in the Th2 cytokine [Fig 2.8a, b and c], an apparent decrease in IL-4, IL-5 and IL-13 levels was observed in T-1, T-2 and T-3 regimens compared to PA group [Fig 2.8a, b, c]. No significant difference was observed in the Th1 cytokines, but an increase in IL-2 and TNF-α was observed compared to naïve group [Fig 2.8 d, e, f, g]. An increase in Th1 cytokines and suppression of Th2 cytokine in PU administered groups can be correlated with the increase in peanut-specific IgG2a antibody levels, as shown in Fig 2.6f. Thus,

cytokine data from these groups further strengthens PU acting as an immunomodulatory compound in allergy-induced mice.

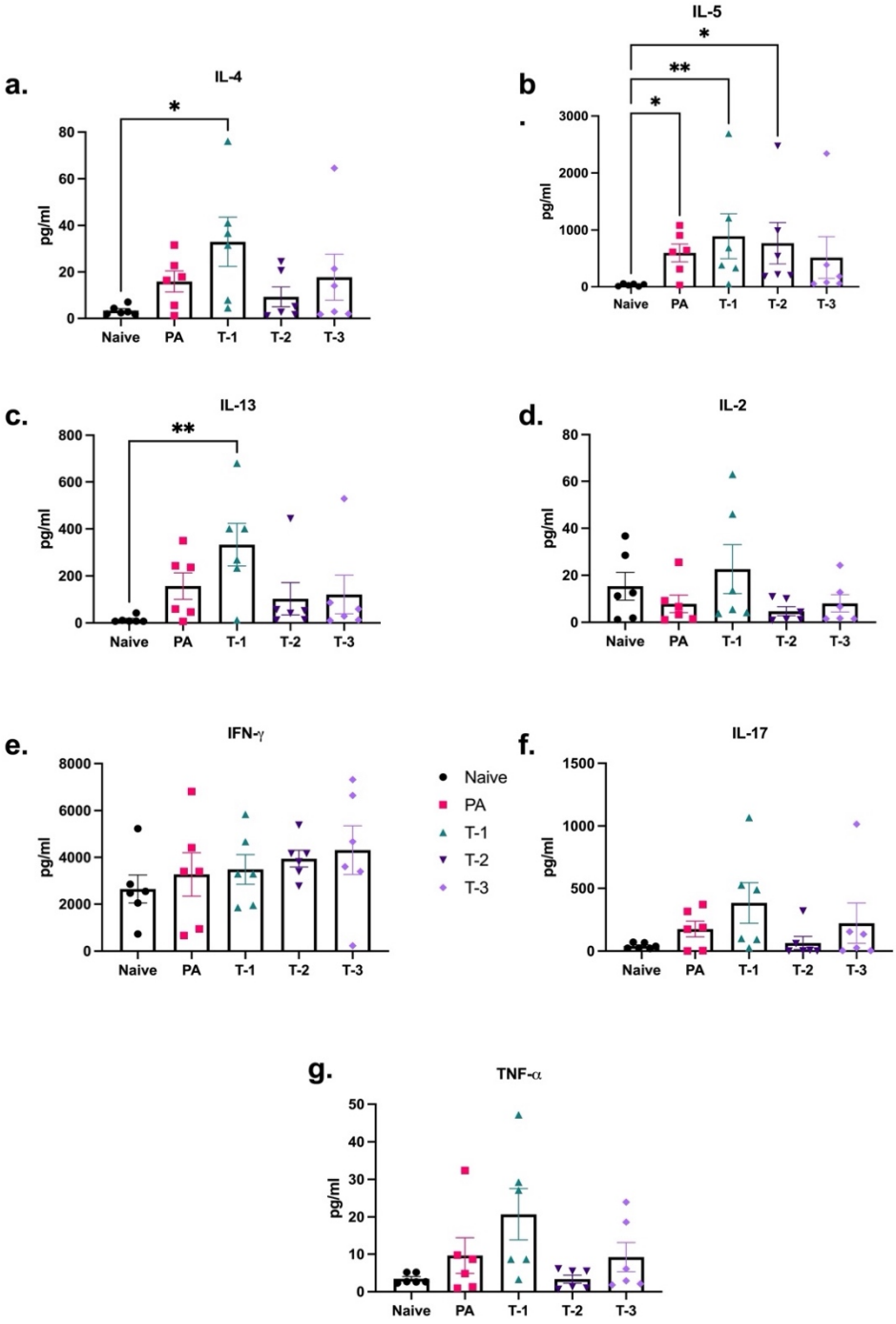

Fig 2.7: Cytokine concentrations after *ex vivo* activation of cultured splenocytes in the crude ulvan biomass group, determined by MILLLIPLEX multiplex assay. Spleens were aseptically excised, and splenocytes were later cultured from all groups of mice. Splenocytes were stimulated using Con A and peanut extract for 72 hrs, and the supernatant was collected to measure Th1/Th2 cytokine levels. (Con A data not shown). a. IL-4, b. IL-5, c. IL-13, d. IL-2, e. IFN-γ, f. IL-17 and g. TNF-α concentration, as represented in pg/ml. Data are represented as mean ± SD of pg/ml of concentration with n = 6 mice/group. Statistical analysis on the data was performed using the Kruskal-Wallis test. *$p < 0.05$ and **$p < 0.005$ compared to indicated group. *PA, peanut allergy, CB, crude ulvan biomass, T-1, T-2 and T-3, three different regimens were based on the administration period's length.*

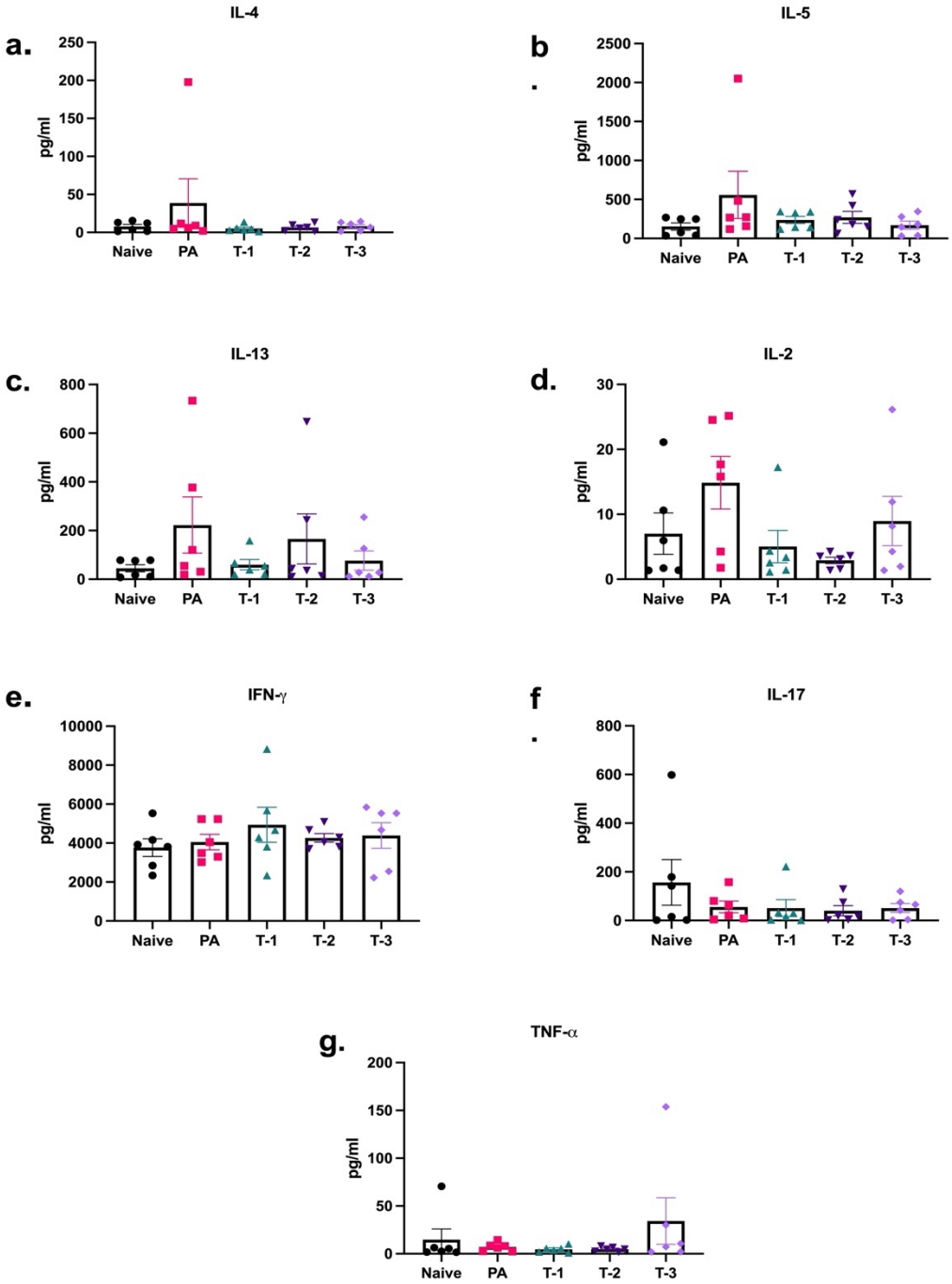

Fig 2.8: Cytokine concentrations after *ex vivo* activation of cultured splenocytes determined by MILLLIPLEX multiplex assay. Spleens were aseptically excised, and splenocytes were later cultured from all groups of mice. Splenocytes were stimulated using Con A and peanut extract for 72 hrs, and the supernatant was collected to measure Th1/Th2 cytokine levels. (Con A data not shown). a. IL-4, b. IL-5, c. IL-13, d. IL-2, e. IFN-γ, f. IL-17 and g. TNF-α concentration, as represented in pg/ml. Data are represented as mean ± SD of pg/ml of concentration with n = 6 mice/group. Statistical analysis was performed using the Kruskal-Wallis test. *PA, peanut allergy, PU, purified ulvan, T-1, T-2 and T-3, three different regimens were based on the administration period's length.*

2.4 Discussion

Food allergy is increasing, with 1 in every 10 children in Australia having some type of food allergy[18]. In a recent survey in the US, one in five adults reported having at least one IgE mediated food allergy[3]. Currently, there is only one immunotherapy for peanut allergy that has been FDA approved for commercial use[19–21]. Thus, there is an urgent need to investigate novel strategies and sources with therapeutic potential to tackle this ever-increasing health problem. Green macroalgae have been documented for regulating various diseases using *in vitro* and *in vivo* systems[6,8,14,15]. However, the potential of green macroalgae to suppress a Th2 response in an allergy model is not yet thoroughly investigated. Therefore, this study was set out to investigate the bioactive potential of the crude ulvan biomass and purified ulvan extracted from green macroalgae *U. ohnoi* in a murine model of peanut allergy. Previous studies have demonstrated that sulphated polysaccharides extracted from marine algae can serve as functional food for nutritional purposes and bioactive compounds for various allergic disorders[7,22–24]. This study utilised female BALB/c mice to induce peanut allergy via oral administration of food-grade peanut extract. Once the mice were sensitized to peanuts, a treatment regimen was applied, where small doses of crude biomass or purified ulvan were orally fed to the mice to investigate its allergic suppressive properties. The group treated with crude biomass showed an increase in the Th2 cytokine response compared to the untreated peanut allergy group. By contrast, the purified ulvan treated group showed marked but statistically non-significant suppression of the Th2 cytokines. Such response could indicate that the crude biomass of green algae may contain other constituents in conjunction with polysaccharide content that may have a Th2 stimulatory property.

In this study, we faced a major caveat with the peanut allergy BALB/C mouse model. Although the sensitization and challenge regimens were performed according to standard protocols, the induction of a Th2 response and IgE antibody production was lacking. As seen with the untreated peanut allergy group, the antibody response was inadequate, and peanut specific IgE antibodies were below detectable levels, as seen by ELISA. This was confirmed by repeating the whole trial under identical conditions. The

sub-optimal response affected our analysis of the Th2 cytokine suppression using purified ulvans.

Insufficient levels of peanut-specific antibody titre of sIgG, sIgG1 and sIgG2a were observed in the serum, suggesting that mice were not adequately sensitized. Previous studies have demonstrated that allergen can be captured by the circulating IgG antibodies, thereby blocking the allergen presentation and suppressing hypersensitivity response by blocking IgE antibody cross-linking on mast cells and in turn preventing the degranulation from initiating the reaction cascade[25–29] .

There are several factors which may have affected the establishment of the peanut allergy model. First, BALB/C mice were used in this model, which is an established strain in allergy research[30,31]. However, it was shown that BALB/C mice elicit a more robust cytokine response than C3H/HeOuJ mice but elicit a weaker antibody response [32,33]. This may have been an important factor in the low antibody response in our study. Secondly, the oral route was chosen for sensitization of the mice using peanut extract, and cholera toxin was used as an adjuvant. This was done to mimic the natural route of sensitization for food allergy and orally treated with the marine algal compounds. However, the literature suggests that the intraperitoneal route gives a stronger cytokine and antibody response[32,34]. Therefore, the selection of the oral route may also have played a role in the poor establishment of the allergy model. Finally, the model was established in a clean SPF facility. A recent review by Dobson *et al.* has pointed out the disadvantages of maintaining a 'clean' environment for animal models for inflammatory and trauma models[35]. The influence of different facilities on outcomes of an allergy model has to be yet investigated.

In summary, the purified ulvans from the marine algae, *Ulva ohnoi,* showed Th2 cytokine suppression and increased IgG production in case of purified ulvan feeding. Although the antibody response was inadequate in this study, it may have been caused due to factors that affected the setup and establishment of the peanut allergy animal model. Further investigations need to be conducted investigating the effects of route of sensitization and different types of housing facilities and their effects on the outcomes on an animal model of food allergy.

2.5 Summary

Our study failed to establish a sustained peanut-based allergic response in the mouse model, and no significant changes in Th2/Th1 cytokine levels were observed. Based on the inadequate peanut-allergic response in mice and other contributing factors, we concluded that the biological efficacy of crude biomass and purified ulvan could not be established.

Although not significant, a trend in the data was observed where crude biomass elicited Th2 skewed response, whereas purified ulvan elicited a Th2 suppressing response. However, no conclusive evidence was established because of a lack of statistical power. Some notable caveats noted in the allergy-based mouse model establishment could be the length of the immunisation protocol, route of sensitisation, type of housing facility or changes in the microbiome. These factors were later investigated in the following chapter for establishing an adequate model of peanut allergy in mice.

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Chapter-3

Impact of housing and route of sensitisation on a mouse model of peanut allergy

Publication in progress:

Impact of housing facility and route of immunisation on a BALB/c model of peanut allergy, and associated effects on the gut microbiota.

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3.1 Introduction

Food allergy is a type-I hypersensitive immune reaction to food antigens that triggers a series of immediate physiological and immune responses that can lead to anaphylaxis and death[1]. In addition to the clinical studies, current studies utilise murine models as tools to study food allergy and anaphylaxis developed against specific food proteins[1,2]. The incidence of food allergy is mainly caused by the 'big-eight' food groups, including peanut, tree nuts, shellfish, fish, egg, milk, wheat, and soy and are steadily increasing[1]. Chapter 2 investigated the safety and efficacy of an ulvan extract, and a purified polysaccharide isolated from green macroalgae *U. ohnoi* in a mouse model of peanut allergy. However, our study was unsuccessful to establish a sustained peanut-based allergic response in the mouse model, and no significant changes in Th2/Th1 cytokine levels were observed. Our observations from chapter 2 indicated that the possible reasons for an unsustained allergic response could involve several factors such as the length of the immunisation protocol, route of sensitisation, type of housing facility or changes in the microbiome. Current research in the design/discovery and pre-clinical development of therapeutic candidates for treating human diseases relies on small animal models, in addition to *ex vivo* human cell models[2,3]. The standardisation and validation of the animal model for food allergy are essential to achieve reproducible clinical and immunological responses[3,4]. This enables the selection of the best biological or immunotherapeutic (antibody, peptides, proteins or small molecules) candidates for potential clinical translation[5–7].

Murine models have enhanced our understanding of the mechanisms underlying allergic sensitisation to food allergens and response to challenge. Current models are established using different mouse strains, including BALB/c, C3HeJ, and C57BL/6, among many others[1,2], reporting challenges in identifying susceptible strains and optimal sensitisation routes, with or without adjuvants, that best resembles human pathology[8,9]. Choosing appropriate mouse models[10,11] and specific routes of immunisation[12] are particularly important since they significantly impact the severity of pathological, cellular and humoral responses. The recent advances in understanding the interactions between the host microbiome and the immune system have shed new light on the importance of animal housing conditions in modulating responses to disease models[13–18]. Variations in the gut microbiota composition, even subtle, could play an

essential role in skewing cell differentiation and immune response towards promoting a wide range of pathophysiological and immune phenotypes in animal models of food allergy[19,20]. However, it is not clear whether the housing conditions could influence the development of a food allergy mouse model using standard procedures. We, therefore, hypothesise that pathophysiological and immune responses of a food allergy mouse model are impacted by the type of housing facility and route of sensitisation.

In this study, we established four BALB/c mouse models of peanut allergy using two different routes of immunisation, housed in two different facilities [i.e., conventional, or specific pathogen-free (SPF)]. Disease pathology, humoral and cytokine responses were compared as a function of route of sensitisation and facility type. In addition, the gut microbiome was assessed in mice from these four groups. A significant difference was observed in the clinical response such as body temperature in the allergy models between the two facilities. Furthermore, differences in specific gut microbial species from the genus *Akkermansia*, *Muribaculum* in SPF facility and *KNHs209* in the conventional facility were also observed. Our study highlights the importance of the infrastructure environment in shaping the microbiome and the immune response when designing and establishing animal models of food allergy.

3.2 Materials and methods

3.2.1 Peanut allergy model

Female, specific-pathogen-free (SPF), 5-6 weeks old BALB/c mice (total n=36; n=6 per group) were obtained from the Australian Institute of Tropical Health and Medicine (AITHM) breeding facility at James Cook University, Townsville, Australia. The mice were maintained on a 12 h light/dark cycle in individually ventilated cages (Tecniplast, NSW). Drinking water and specialised food pellets (Speciality feeds, Australia) with no nuts or cross-reactive allergen sources were fed to the mice ad libitum. Mice were assessed for pathogen-free state using sentinels by confirming the absence of a certain set of bacteria, fungi, ectoparasites, endoparasites, non-pathogenic protozoa and viruses (Cerberus Sciences, Adelaide, Australia). Experiments in this study were carried out following the recommendations from an independent ethics committee for animal experimentation (the Animal Ethical Committee of James Cook University, Townsville, Australia, and Ethics ID: A2426).

The mice were acclimatised for 2 weeks in the conventional or SPF facility before initiating immunisations. Mice were sensitised using intraperitoneal (IP) or oral routes at days 0, 7 and 14 with 0.5 mg peanut with 2 mg alum for the IP group or 10 mg peanut with 20 ug cholera toxin B [Fig 3.1]. On days 21 and 28, mice were orally challenged with 50 mg or 100 mg of peanut extract in the IP or oral sensitised group respectively [Fig 3.1]. Naïve mice received 150 µl of phosphate buffered saline (PBS) by oral gavages or IP injection. Symptomatic and diarrheal scoring was performed for 60 mins after each challenge. The temperature was measured using a ThermoScan 7 thermometer (Braun, Australia) 30 mins after the administration of the last challenge dose. Body weights were measured weekly for four weeks. The antibody levels in serum were measured from blood samples collected every week post sensitisation and challenge. On day 29, the mice were euthanised, gut tissues and spleen were collected and stored in RNA ladder and culture media respectively for histology or cytokine analysis. Faecal samples were collected from each cage (housing 3 mice/cage) every week, snap frozen, and stored at -80°C until further analysis.

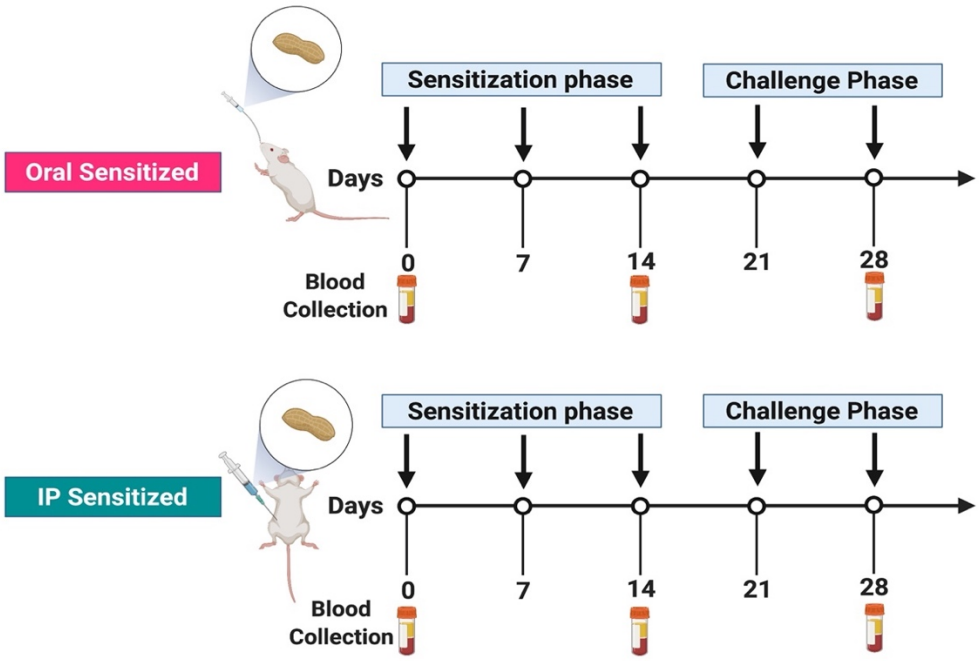

Fig 3.1: BALB/c peanut allergy model setup for comparing impact of route of sensitisation and mouse facility on clinical and immunological outcomes. Mice were housed in a conventional and a SPF facility before receiving sensitisation dose orally or intraperitoneally (IP) on days 0, 7 and 14, using 0.5 mg or 10 mg of peanut extract, mixed with 2 mg alum or 20 µg cholera toxin B (CTB), respectively. Sensitisation was followed by oral challenge on day 21 and 28, in both oral and IP sensitised models using 50 mg and 100 mg of peanut extract respectively.

3.2.2 Reagents

Peanut extract was prepared from food grade peanut flour from Golden Peanut Speciality Products Division, Georgia, USA (www.goldenpeanut.com). Briefly, the peanut flour was mixed in 1× PBS and left overnight at 4°C on a shaker the day before use. Cholera toxin (CT) and alum adjuvant was purchased from Sigma Aldrich (Melbourne, Australia). Mouse IgG, IgG1 and IgG2a antibody used for detecting peanut-specific antibody titre were obtained from Life Technologies (Mulgrave, Victoria, Australia). Coating buffer for ELISA was prepared using sodium carbonate (0.2M) and sodium bicarbonate (0.2M) with pH-9.6 using double distilled water. Washing buffer (PBS-tween) solution for ELISA was prepared by mixing 1X PBS with 0.05% tween solution. Blocking solution for ELISA was prepared using a 1:9 ratio of 10X casein solution (company, country) in PBS-tween solution. Antibody buffer for ELISA was prepared using the .05X casein solution. TMB solution (Life Technologies, Melbourne, Australia) was used to develop the ELISA reaction.

3.2.3 Antibody and cytokine response

Serum based peanut-specific IgG, IgG1, and IgG2a (Life Technologies, Victoria, Australia) were quantified using our in-house ELISA protocol and total IgE levels (Biolegend, Australia) quantified using a commercial ELISA kit according to the manufacturer's protocol. Gene expression of Th1 and Th2 cytokines were measured in the duodenum tissue using the RT2 Profiler innate and adaptive immune response PCR Array for mouse (PAAM-52Z; Qiagen, Victoria, Australia) according to the manufacturer's protocol. The expression levels were quantified relative to the values obtained for the housekeeping gene GAPDH. Data analyses was performed using web based Genegobe Qiagen analysis software designed for PCR array analysis.

3.2.4 Histology to assess tissue infiltration and mast cell degranulation

The intestinal tissue specimens were stored in the 4% paraformaldehyde overnight and were later transferred to 80% ethanol for histological examination. An increasing concentration of ethanol were used to dehydrate the fixed intestinal tissue specimens and were later transferred to benzene, benzene-paraffin, and paraffin. For staining

purposed, intestinal tissues sections (5µm) were deparaffinised using xylene, rehydrated through ethanol leading to water gradient and stained for Hematoxylin and eosin (H&E) stain for analysing anatomical structural changes or infiltration. Toluidine blue staining was performed to analyse mast cell numbers in mucosa and villus in the duodenum. The numbers of mast cells were determined per villus by randomly selecting ten villi in ten regions of interests on each section of intestine. Degranulated mast cells were counted based on the loss of cellular structure integrity and visible granule secretion from mast cells in the tissue. Independent cell counting and measurements were performed by three individuals and average results recorded for each mouse.

3.2.5 Microbiome analysis

3.2.5.1 DNA extraction and 16S rRNA gene amplification and sequencing

Faecal samples were collected in DNAse and RNAse free tubes and stored at -80°C. Total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen, Germany) following the manufacturer's instructions and following the protocol of Zhou et al [21]. Bacterial communities from faecal samples were investigated by sequencing 16S rRNA gene amplicons as described previously[22]. The primers 341F and 785R were used to amplify the V3-V4 regions of the 16S rRNA gene[22]. The reaction mixture (50 µl total volume per sample) consisted of Econotaq® PLUS GREEN 2X master mix (Lucigen) (25 µl), Ambion® nuclease-free water (17 µl), the primer pair 341F and 785R (1.5 µl of each; 10 µM) and DNA template (5 µl). The PCR program consisted of an initial denaturation at 94°C (2 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s) and extension at 72°C (40 s), and a final extension of 72°C (7 min). PCR products were then quantified using gel electrophoresis. Paired-end sequencing (2 x 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics (RCG), UNSW on an Illumina MiSeq platform as per the MiSeq System User Guide (Illumina 2013).

3.2.5.2 16S rRNA gene sequence analysis

Sequence data were initially quality-filtered and trimmed using TRIMMOMATIC version 0.36 truncating reads if the quality dropped below 20 in a sliding window of 4

bp[23]. USEARCH version 11.0.667[24] was used for further processing as described by Wemheuer and Wemheuer[25] to merge, and quality-filter sequencing reads, excluding reads with < 250 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into amplicon sequence variants (ASV) using the UNOISE3 algorithm[26] implemented in USEARCH. ASVs represents unique bacterial entities and roughly are equivalent to species or strains. Chimeric sequences were removed de novo during clustering and subsequently in reference mode using UCHIME[27] with the GTDB database v86 (https://data.gtdb.ecogenomic.org/releases/release86/) as a reference. ASVs were then taxonomically classified (i.e., assigned a likely taxonomic name) by BLASTN[28] against the GTDB database. All non-bacterial ASVs were removed along with non-BLAST aligned and singleton ASVs. Finally, processed sequences were mapped on ASV sequences to calculate the count distribution and counts of each ASV in every sample. Only ASVs occurring in more than two samples were considered for further statistical analysis.

3.2.6 Community and statistical analysis

Rarefaction curves were generated using the rarecurve function in vegan[29] and used to determine if a complete representation of the sample's microbiome had been achieved given the sequencing effort. Prior to further analysis the numbers of sequences were standardised across samples to account for different sequencing depths by randomly subsampling each sample to the lowest number of sequences counts obtained for any given sample (i.e., 10,507 counts). Bacterial alpha-diversities (i.e., ASVs richness and Shannon's diversity) were calculated in R (version 3.5.3) using the rrarefy function in the vegan package for community ecology analysis[29]. A one-way ANOVA test in GraphPad Prism 8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups with a p-value <0.05 being significant.

The effects of 'Facility' and 'Treatment' on the microbial community structure were tested by permutational multivariate analysis of variance (PERMANOVA) and pairwise comparisons with 9,999 random mutations. Bray-Curtis similarity distances were

calculated using square-root transformed ASV abundances and the resulting similarity matrix was visualised using non-metric, multi-dimensional scaling (nMDS). The R package vegan and pairwise.adonis2[30] was used and the pairwise test p-values were adjusted using a Benjamini & Hochberg method[31]. To check if any observed statistical difference was caused by different dispersions in data, a test for homogeneity of multivariate dispersions (PERMDISP) was performed[32]. A *p*-value <0.05 was considered significant. All analyses and statistics were performed under R version 3.6.3 (R Core Team, 2019). 'Facility' was set with "SPF" and "conventional"; and 'Treatment' was set with "Naïve", "IP sensitised" and "Oral sensitised" as fixed factors.

3.3 Results

3.3.1 Systemic response to peanut challenge

On day 28, mice from both SPF and conventional facilities were challenged with peanut extract [Fig 3.2]. Body temperature and symptom scores were measured every 10 mins for up to 1 hour. A decrease in body temperature was observed between 10- and 20-mins post challenge for all test groups. There was no statistical support for a change in body temperature for both the IP and oral sensitised groups from the SPF facility 20 mins after challenge compared to the control group [Fig 3.2].

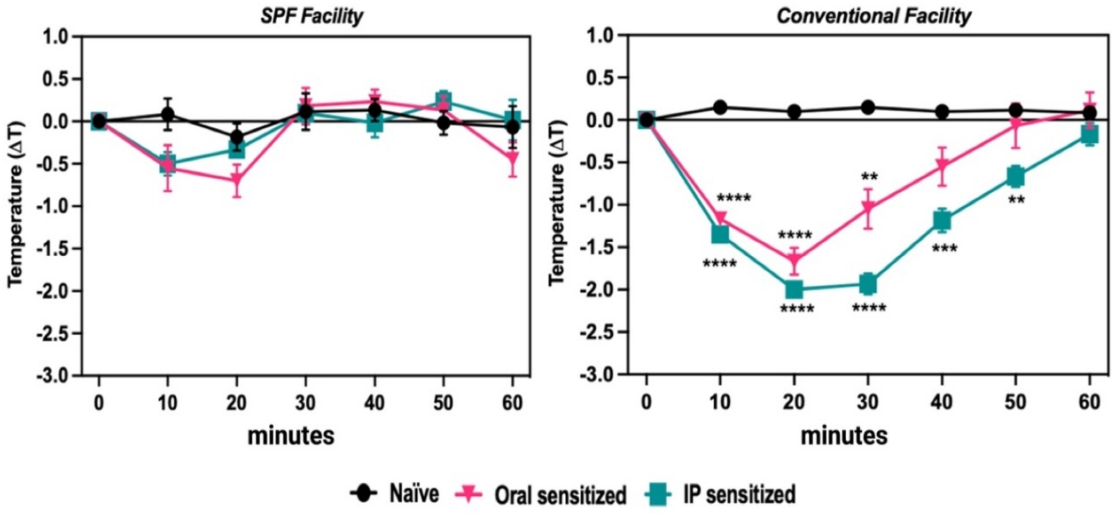

Fig 3.2: Body temperature was measured for 1 h every 10 mins after the last challenge on day 28 in naïve, oral sensitised and IP sensitised mice. Difference in the temperature between naïve and challenged mice challenge is shown. Data are plotted as mean values ± SEM. (Two-way ANOVA with Tukey's multiple comparison test) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

In contrast, a significant decrease in body temperature was observed for both IP and oral sensitised mice from the conventional facility up to 30 mins post-challenge [Fig 3.2; $p \leq 0.0001$]. There was a significant drop in body temperature for the IP sensitised group compared to naïve group, causing a steeper and prolonged decrease in temperature for up to 50 mins (p-value <0.01). Furthermore, no significant changes were observed in the naive groups in either SPF or conventional facilities.

3.3.2 Humoral and cytokine responses

Peanut-specific IgG, IgG1, IgG2a and total serum IgE were measured on day 0 and day 28 to assess and compare induction of allergic sensitisation in mice between the two different facilities. A significant increase in IgE antibodies was observed on day 28 for the IP sensitised mice in SPF and conventional facilities compared to oral sensitised mice [Fig 3.3; $p \leq 0.001$]. There was no statistical support for any differences in IgE antibody titres in the IG sensitised mice compared to the naïve group. No facility-specific differences were found in the IgE antibody responses. In contrast, peanut-specific IgG and IgG1 antibody levels were significantly higher in the IP sensitised mice on day 28 in both SPF and conventional facilities [Fig 3.4A, B; $p \leq 001$ $p \leq 0.0001$]. The oral sensitised groups did not elicit a higher IgG or IgG1 response compared to the naïve animals [Fig 3.4A, B]. There was no significant increase in the IgG2a response compared to the naïve group; however, the response in the oral sensitised group followed a similar (higher) trend on day 28 when compared to IgG and IgE responses [Fig 3.4C].

To measure the cytokine response, fold changes were calculated for Th2 (IL-4, IL-5, IL-13) and Th1 (IFN-γ, IL-2, TNFα) specific cytokines in gut tissues of IP and oral sensitised mice in relation to the naïve mice in both housing facilities. Th2 and Th1 cytokines levels were upregulated for IP and oral sensitised mice in the conventional facility, while Th2 and Th1 cytokines were downregulated in the SPF facility [Table 3.1]. The IP sensitised group showed a higher upregulation of Th1 and Th2 cytokines as compared to oral sensitised mice, in the conventional facility. There was a significant increase in IL-4 response in IP sensitised group compared to naïve group in the conventional facility (p<0.05).

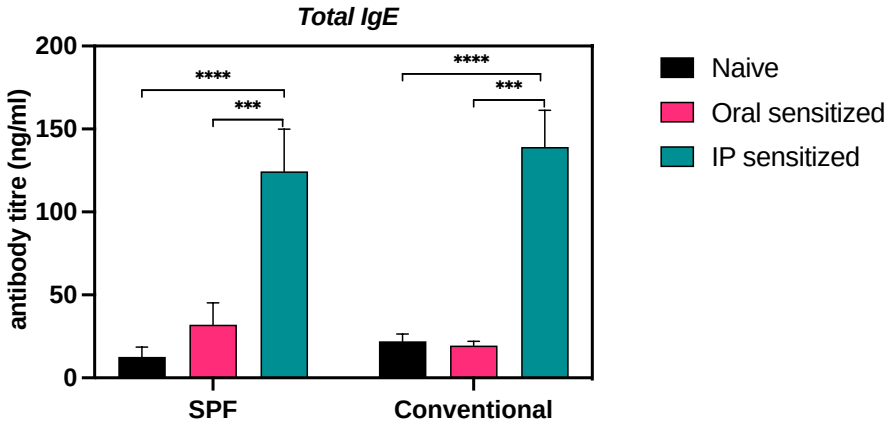

Fig 3.3: Total IgE in serum samples was measured in naïve, oral and IP sensitised mice. Data are expressed as optical density (absorbance at 450 nm). (Two-way ANOVA with Tukey's multiple comparison test) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

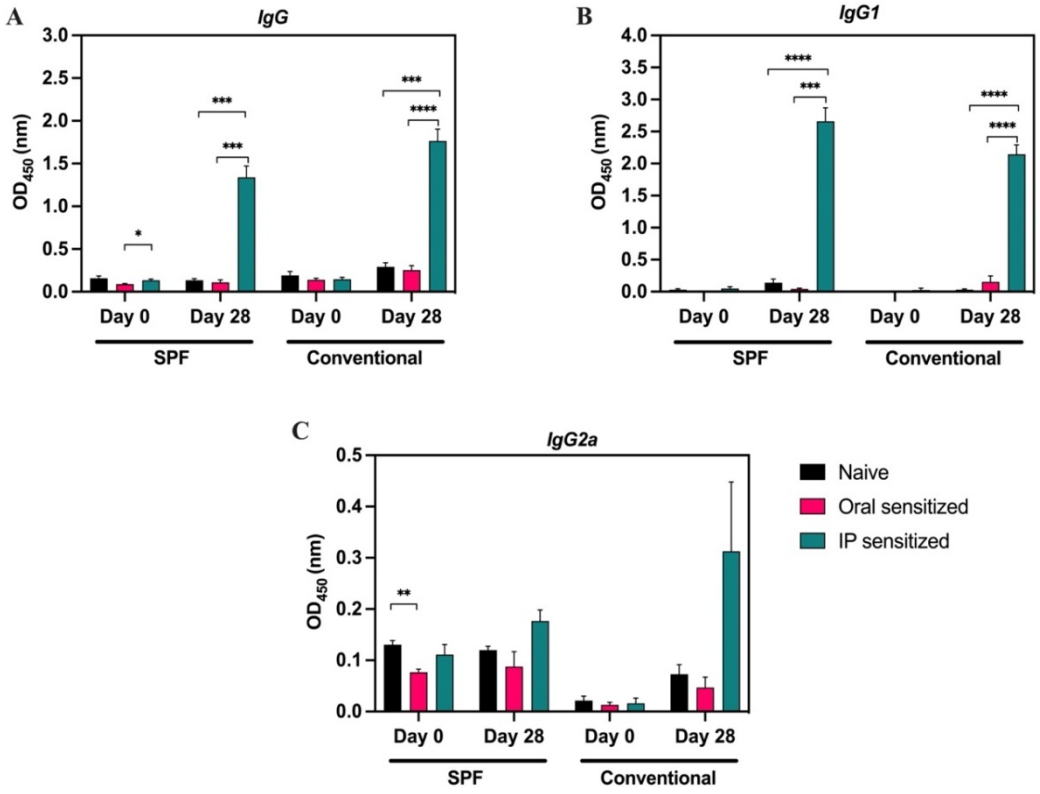

Fig 3.4: Peanut-specific antibody response in sera of naïve, oral and IP sensitised mice housed in SPF or conventional facility. Levels of peanut-specific antibodies (A) IgG, (B) IgG1 and (C) IgG2a in SPF and conventional facilities (days 0 and 28) was measured by ELISA. Data are expressed as optical density (absorbance at 450 nm). (Two-way ANOVA with Tukey's multiple comparison test) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

Table 3.1: qPCR gene expression analysis of Th1 and Th2 specific cytokines in mouse gut (duodenum) tissue. Data is displayed as fold change in comparison to the naïve control mice. Statistical analysis was performed using student t-test with $p > 0.05$ considered significant.

Cytokine	Conventional facility			
	Oral sensitised		IP sensitised	
	Fold change	*p-value*	Fold change	*p-value*
Th2				
IL4	1.54	0.392148	3.34	**0.032791**
IL5	2.56	0.328166	3.59	0.153946
IL13	1.19	0.653051	3.28	0.173348
Th1				
IFN-γ	2.51	0.328849	3.52	0.149059
IL2	2.65	0.330821	4.24	0.147494
TNF-α	2.26	0.343476	3.47	0.126555
Cytokine	**SPF facility**			
	Oral sensitised		**IP sensitised**	
	Fold change	***p-value***	**Fold change**	***p-value***
Th2				
IL4	0.16	0.103254	0.12	0.092696
IL5	0.26	0.233019	0.24	0.218652
IL13	0.3	0.234841	0.21	0.215568
Th1				
IFN-γ	0.57	0.731667	0.24	0.218652
IL2	0.26	0.233013	0.24	0.218652
TNF-α	0.19	0.196885	0.17	0.188931

3.3.3 Gut tissue resident mast cell degranulation

Mast cell degranulation was assessed using toluidine blue staining and histological scoring of mast cells in the duodenal tissue of mice for all treatment groups and housing facilities [Fig 3.5 A, B]. The total mast cells found in gut tissue were significantly higher in the IP sensitised group ($p \leq 0.01$) and oral sensitised mice ($p \leq 0.05$) compared to the naïve group in the SPF facility, however not in the conventional facility [Fig 3.5 C, D]. Interestingly, the degranulated mast cell count was significantly higher in the IP sensitised mice ($p \leq 0.0001$) and oral sensitised mice ($p \leq 0.01$, $p \leq 0.05$) in both facilities compared to naïve mice [Fig 3.5 C, D]. There was no statistical support for changes observed in mast cell degranulation between the two housing facilities.

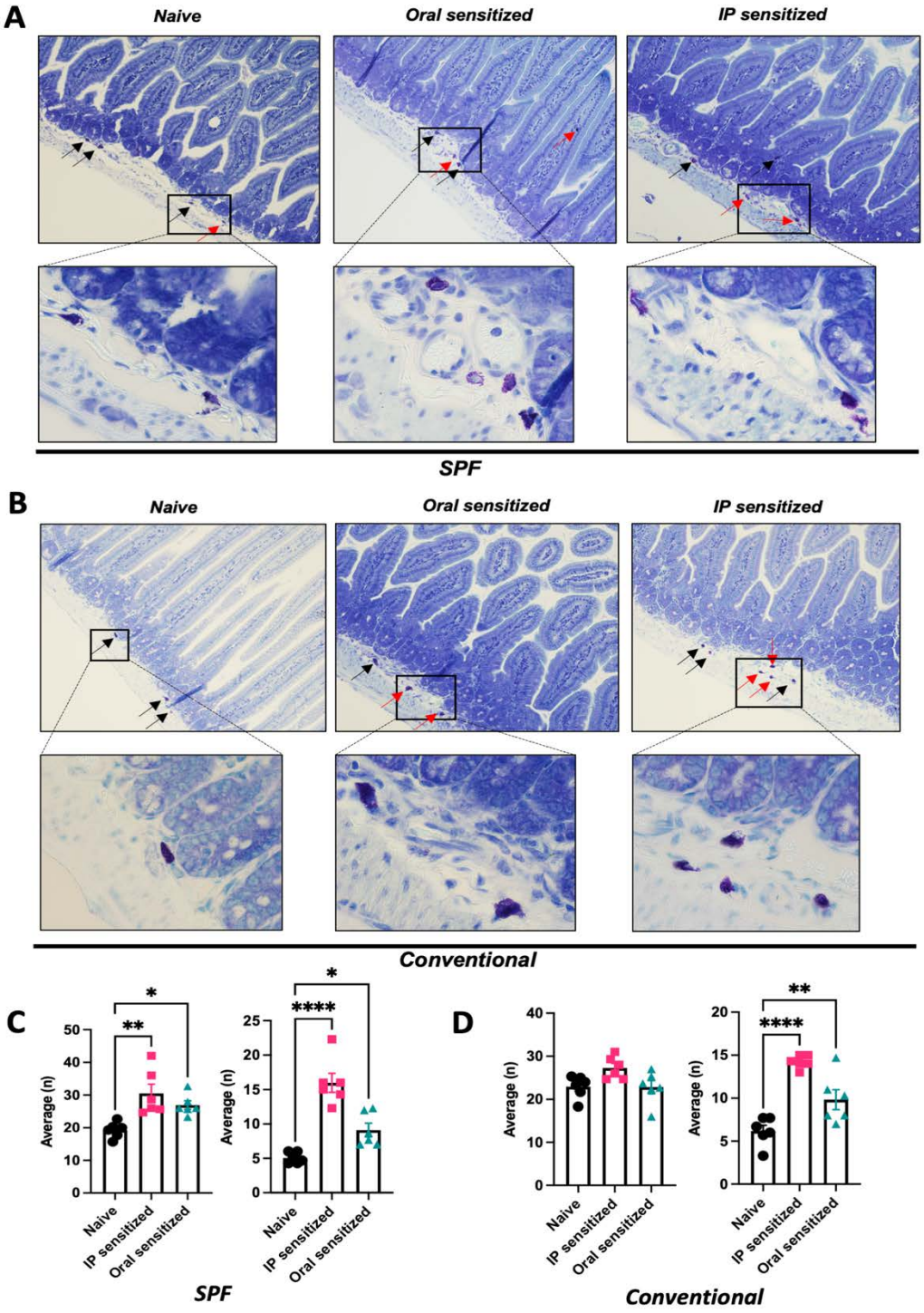

Fig 3.5: Histology score of total number of mast cells and degranulated mast cells in the duodenum of mice housed in SPF and conventional facility. Toluidine -blue staining for mast cell population was performed on the intestinal tissue of mice from the naïve, oral sensitised and IP sensitised group in (A) SPF and (B) conventional facilities (C) SPF-total mast cell number [left], SPF-degranulated mast cell number [right] (D) conventional-total mast cell number [left], conventional-degranulated mast cell number [right]. Data presented here is an average of 3 independent measurements. Two-way ANOVA with Dunnett's multiple comparison test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

3.3.4 Bacterial community structure is influenced by facility type and route of sensitisation

16S rRNA gene-based analysis was used to assess bacterial communities. Data was obtained for faecal samples of mice from two different facilities (SPF and conventional) and three different treatments (Naïve, IP sensitised and Oral sensitised) after the challenge phase. The samples from naïve (2), IP sensitised (2) and oral sensitised (1) from conventional facility were lost during the sample processing. After quality filtering there were a total of 299,208 sequences and these were clustered into 609 ASVs. The calculated rarefaction curves based on rarefied and unrarefied data as well as the good's coverage of 94.75 ± 12.99% indicated that the majority of the bacterial community was recovered by the surveying effort [Fig 3.6].

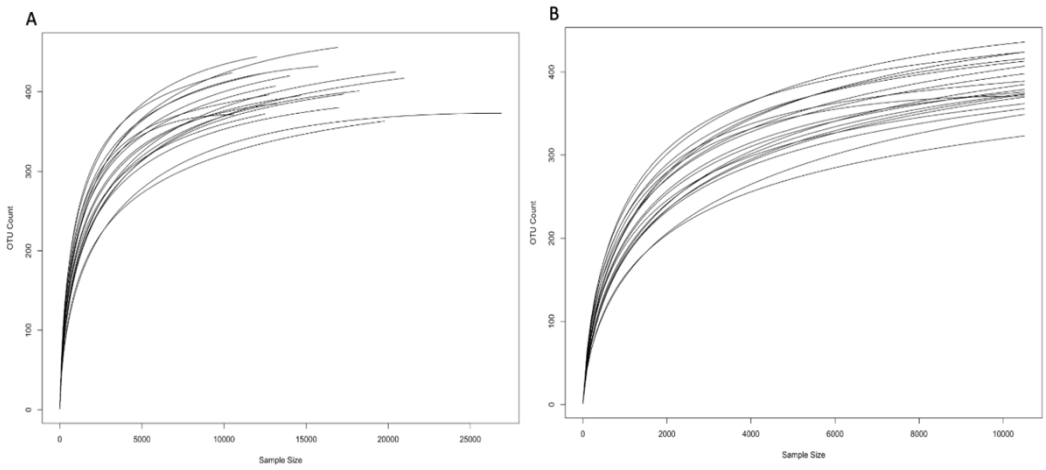

Fig 3.6: Normalised sequence data before and after rarefaction analysis. (10,507 sequences)

Hierarchical clustering demonstrated the bifurcation of the bacterial community structure in BALB/c mice faecal microbiome based in SPF or conventional facility during the challenge phase, except for the Naïve group (SPF) which were clustered closer to the naïve group based in conventional facility. Multi-dimensional scaling (MDS) plot reveals the separate clustering of peanut allergy groups (IP- or oral sensitised) as compared to the respective naïve control groups in the SPF and conventional facilities. Interestingly, the IP-sensitised or oral-sensitised groups resembled each other in the respective facilities [Fig 3.7]. An overall effect of the mouse facility type (PERMANOVA, p = 0.0001) and route of sensitisation

(PERMANOVA, p = 0.0078) was observed for bacterial community structure based on Bray-Curtis dissimilarity, as well as the interaction of the two factors facility and treatment (PERMANOVA, p = 0.015) [Table 3.2]. In the SPF facility, there was statistical support for differences in bacterial community structure between the naive group and the IP sensitised (p = 0.029) and oral sensitised group (p = 0.031) [Fig 3.7, Table 3.3]. In the conventional facility, no significant differences in the bacterial community structure were found between naïve, IP sensitised or oral sensitised groups, possibly due to low number of biological replicates due to loss during sample processing. There was statistical support for differences in the oral sensitised group between the two housing facilities (p<0.05). Overall, these observations indicate a strong effect on the bacterial community structure in a peanut allergy model, based on the facility type and route of immunisation.

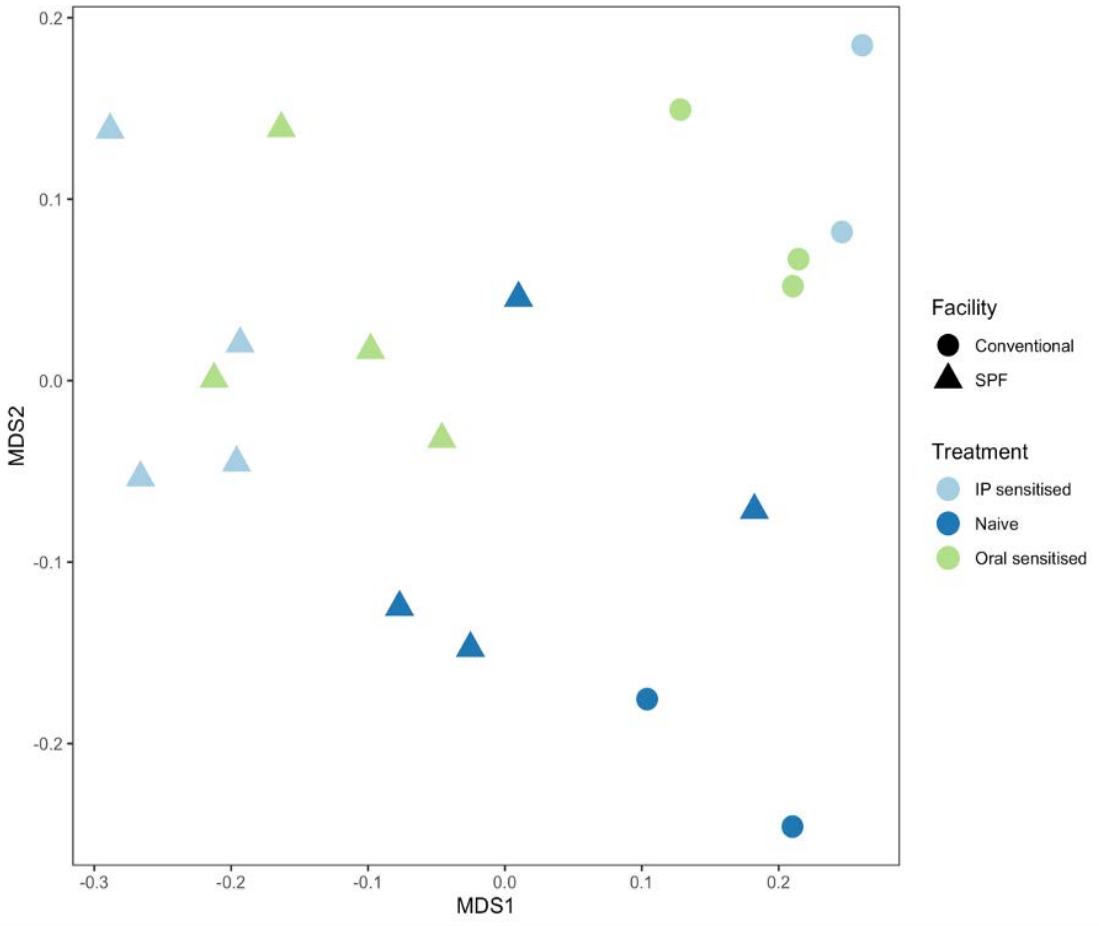

Fig 3.7: Multi-dimensional scaling (MDS) plot of bacterial community structure of murine faecal samples from SPF and conventional facilities between IP-sensitised, oral-sensitised mice and naïve control groups.

Table 3.2: PERMANOVAs based on Bray-Curtis (BC) similarity measure for square-root transformed abundances of all mice faecal samples collected from SPF and conventional facilities, all treatments and induction phases. *p*-values were calculated using 999 permutations under a residual model. Bold indicates statistical significance (at alpha = 0.05).

Source	Df	SumOfSqs	MeanSqs	F.model	R2	Pr(>F)
Facility	**1**	**0.55209**	**0.55209**	**10.3181**	**0.30446**	**0.0001**
Treatment	**2**	**0.29677**	**0.14839**	**2.7732**	**0.16366**	**0.0078**
Facility:Treatment	**2**	**0.26888**	**0.13444**	**2.5126**	**0.14828**	**0.0150**
Residuals	13	0.69559	0.05351		0.38360	
Total	18	1.81334			1.0000	

Table 3.3: PERMANOVAs based on Bray-Curtis (BC) similarity measure for square-root transformed abundances of all mice faecal samples collected from SPF and conventional facilities and all treatments during the challenge phase. *p*-values were calculated using 999 permutations under a residual model. Bold indicates statistical significance (at alpha = 0.05).

Pairs	F.model	R2	*p* value
Conventional facility comparing all treatments			
IP sensitised vs Oral sensitised	1.359929	0.3119154	0.2
IP sensitised vs Naive	5.069774	0.7171055	0.33333333
Oral sensitised vs Naive	3.338734	0.5267194	0.1
SPF facility comparing all treatments			
IP sensitised vs Oral sensitised	0.9204095	0.1329993	0.517
IP sensitised vs Naive	**4.0258667**	**0.4015480**	**0.029**
Oral sensitised vs Naive	**2.3458202**	**0.2810773**	**0.031**
SPF vs conventional facilities			
Naive treatment	2.070632	0.34109	0.1333333
Oral sensitised treatment	**5.715622**	**0.5333915**	**0.023**
IP sensitised treatment	8.931296	0.6906729	0.06666667

3.3.5 Intraperitoneal route of sensitisation shows stronger effect on alpha diversity of bacterial communities than intragastric route in peanut allergy model

Alpha diversity measures (Shannon index and richness) were calculated and compared for differences between naïve or allergy groups or between the SPF and conventional facility [Fig 3.8]. A decrease in diversity and richness was observed for the IP sensitised group

compared to the naïve group in the SPF facility, while an increase was observed for IP sensitised group for the conventional facility. Statistical significance could not be calculated due to loss of biological replicates in the conventional group during sample processing. A significant decrease in richness was observed for the IP sensitised group in the SPF facility compared to naïve (p=0.0167) and oral sensitised groups (p=0.0172) [Fig 3.8 A, B].

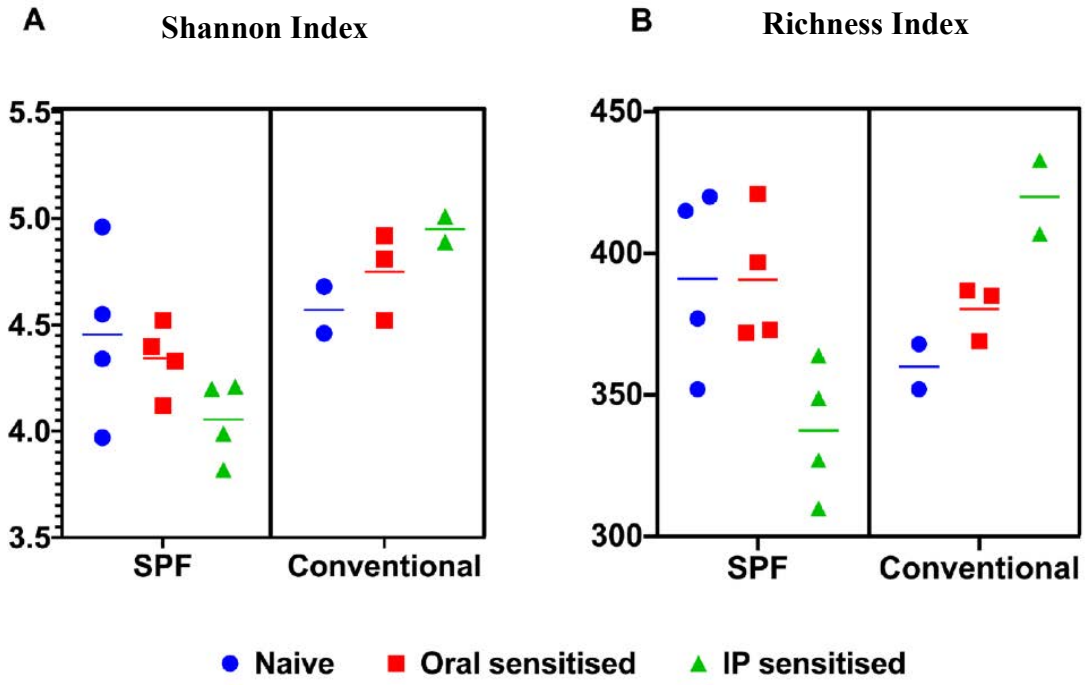

Fig 3.8: Shannon diversity (A) and richness (B) of faecal samples from SPF and conventional facilities for the naïve group and peanut allergy groups that were sensitised intraperitoneally (IP) or via oral route. Tukey's pairwise comparisons test was used to determine the significance between the different groups with a p-value <0.05.

3.3.6 Taxonomic differences in bacterial communities were impacted by mouse facility and route of sensitisation

The faecal samples from both facilities for different treatment groups had most abundance of phyla *Bacteroidota*, *Firmicutes*, *Firmicutes_A* and *Verrucomicrobiota* [Fig 3.9]. Analysis of the bacterial community structure at the class level showed that *Bacilli*, *Bacteroidia*, *Clostridia* and *Verrucomicrobiae* were found to be most dominant in the faecal samples [Fig 3.10]. Analysis of the bacterial community structure at the family level showed that *Muribaculaceae* (class *Bacteriodia*), *Lachnospiraceae* (class

Clostridia), *Oscillospiraceae* (class *Clostridia*) and *Akkermansiacaeae* (class *Verrucomicrobia*) were found to be most dominant in the faecal samples [Fig 3.11].

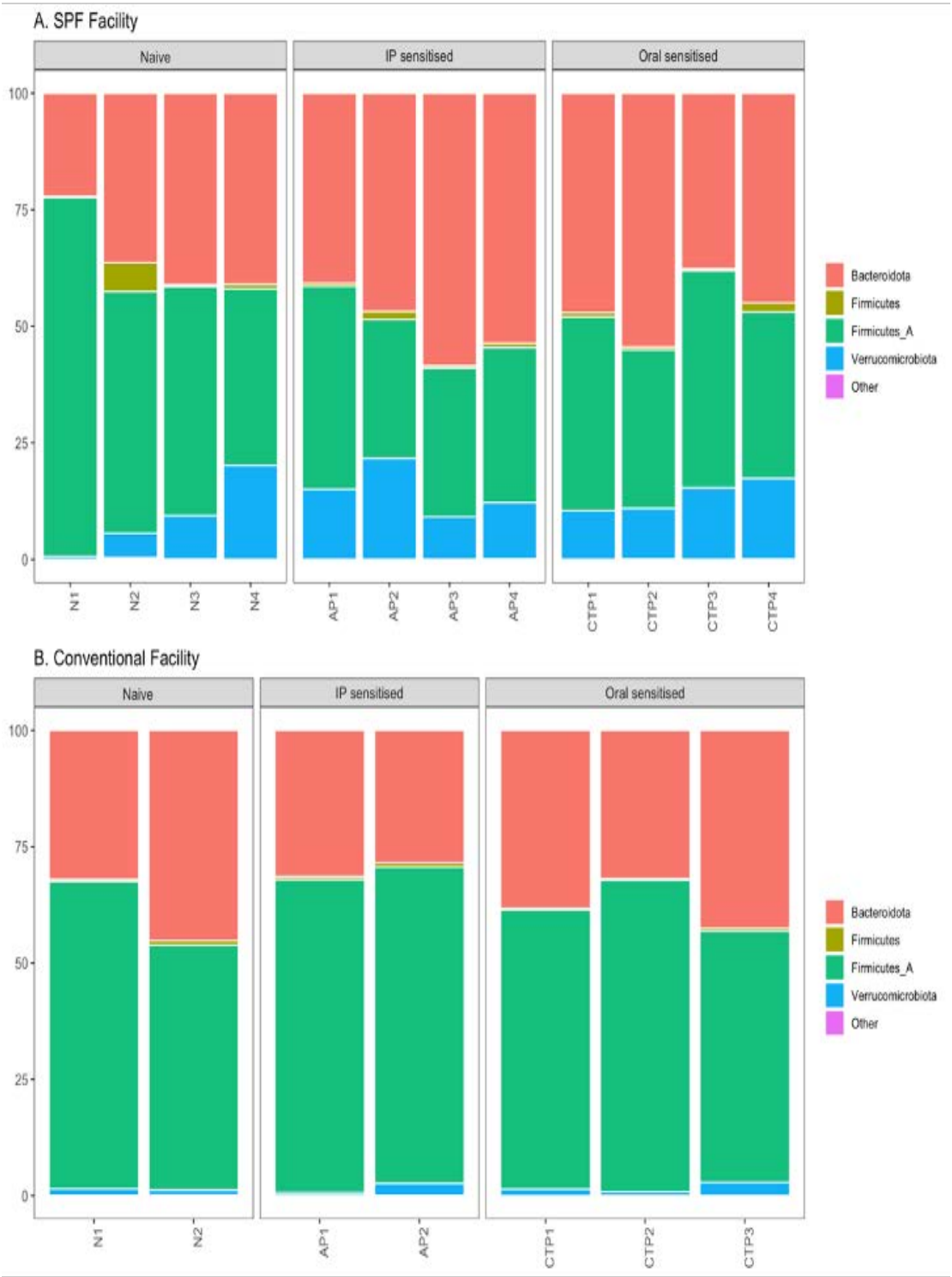

Fig 3.9: Taxonomic profiles of bacterial communities at the phylum level of all faecal samples from the SPF and conventional facilities in naïve, IP sensitised and oral sensitised groups.

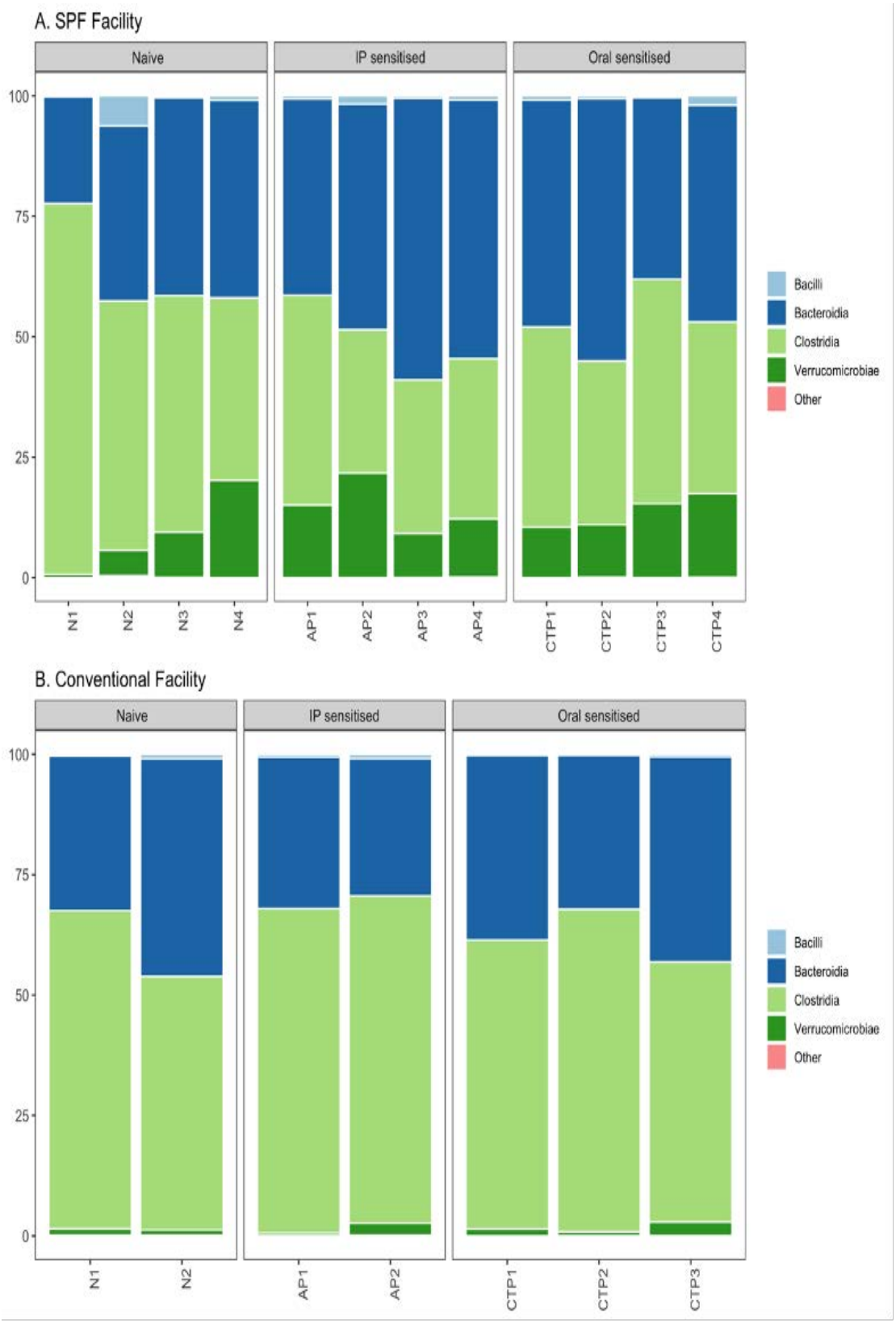

Fig 3.10: Taxonomic profiles of bacterial communities at the class level of all faecal samples from the SPF and conventional facilities in naïve, IP sensitised and oral sensitised groups.

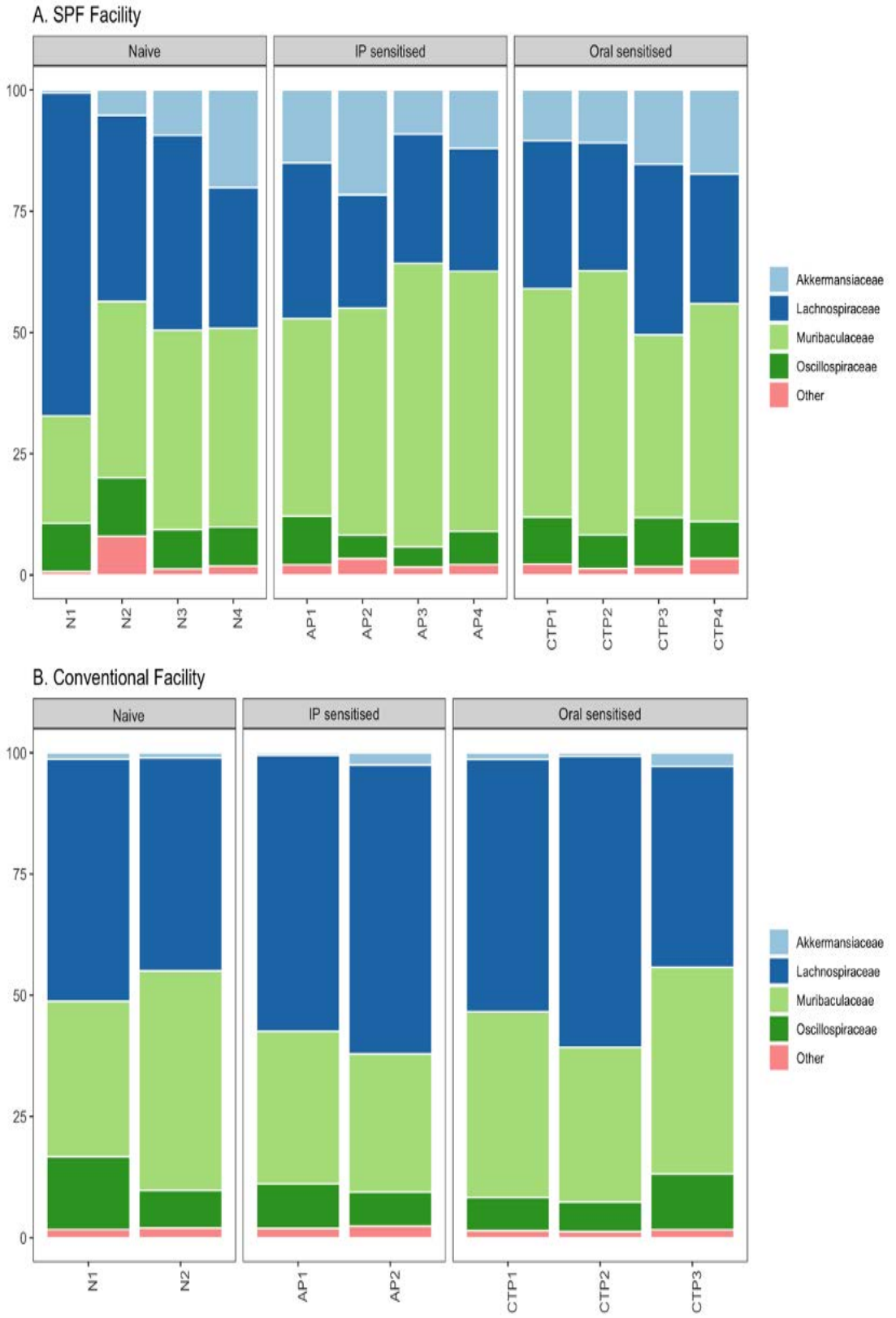

Fig 3.11: Taxonomic profiles of bacterial communities at the family level of all faecal samples from the SPF and conventional facilities in naïve, IP sensitised and oral sensitised groups.

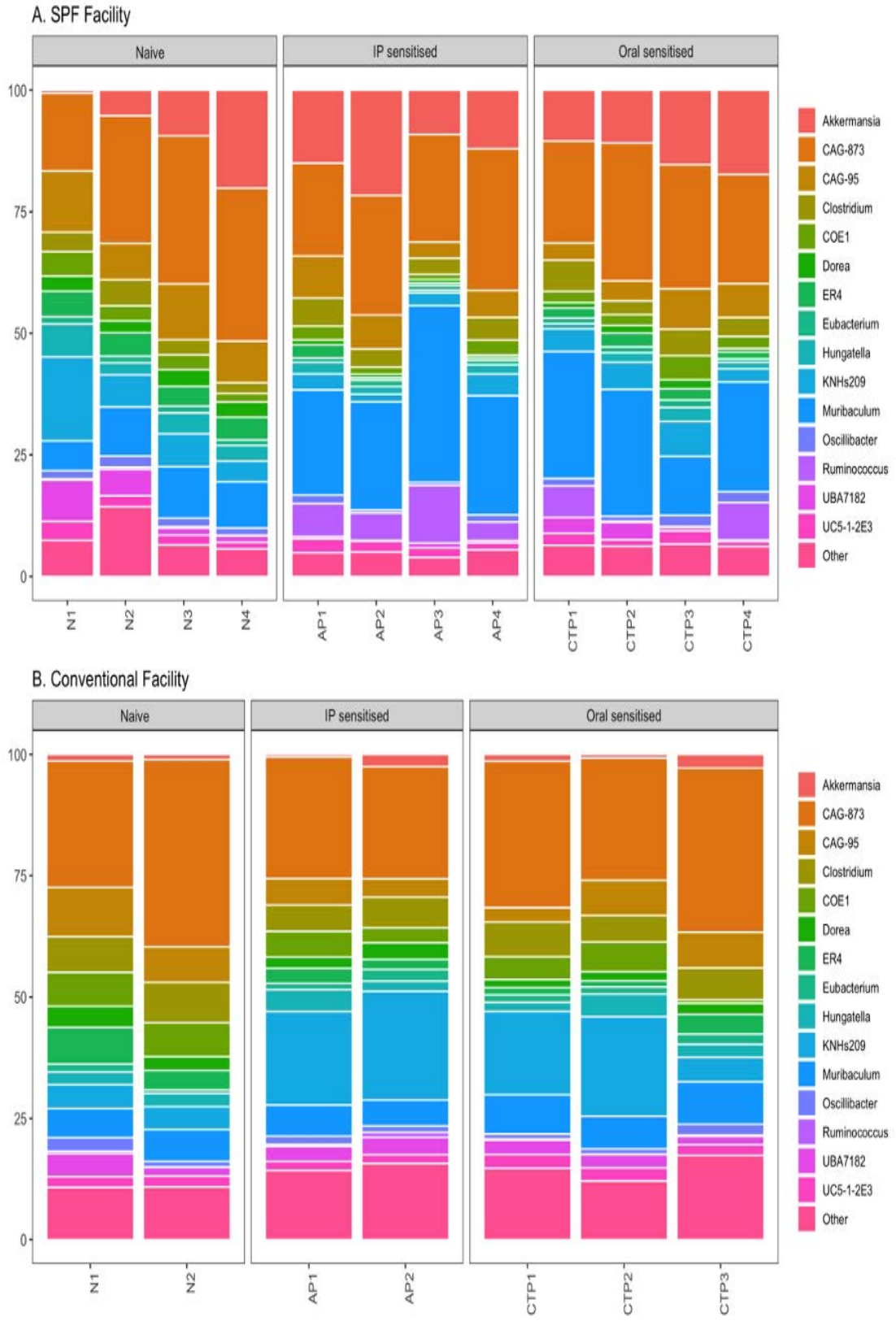

Fig 3.12: Taxonomic profiles of bacterial communities at the genus level of all faecal samples from the SPF and conventional facilities in naïve, IP sensitised and oral sensitised groups.

Analysis of the bacterial community structure at the genus level showed that the *Akkermansia* (family *Akkermansiaceae*), *CAG-873* (family *Muribaculaceae*), *CAG-95* (family *Lachnospiraceae*), *Clostridium* (family *Oscillospiraceae*), *COE1* (family *Lachnospiraceae*), *Dorea* (family *Lachnospiraceae*), *ER4* (family *Oscillospiraceae*), *Eubacterium* (family *Lachnospiraceae*), *Hungatella* (family *Lachnospiraceae*), *KNHs209* (family *Lachnospiraceae*), *Muribaculum* (family *Muribaculaceae*), *Oscillibacter* (family *Oscillospiraceae*), *Ruminococcus* (family *Ruminococcaceae*), *UBA7182* (family *Lachnospiraceae*) and *UC5-1-2E3* (family *Lachnospiraceae*) were most dominant in the faecal samples [Fig 3.12].

An increase in the relative abundance of bacteria from the genus *Akkermansia* and *Muribaculum* was observed in the IP sensitised and oral sensitised samples for the SPF facility (IP sensitised: *Akkermansia*-14.46 ± 5.36%; *Muribaculum*-26.18 ± 6.89%; Oral sensitised: *Akkermansia*-13.50 ± 3.37%; *Muribaculum*-21.71 ± 6.60%) compared to conventional facility (IP sensitised: *Akkermansia*-1.54 ± 1.39%, $p < 0.0001$; *Muribaculum*-5.89 ± 0.73%, $p < 0.0001$; Oral sensitised: *Akkermansia*-1.67 ± 1.05%; *Muribaculum*-7.86 ± 1.02%, $p < 0.0001$). In contrast, an increase in the relative abundance of bacteria from the genus *KNHs209* was observed in the conventional facility (IP sensitised: 20.82 ± 2.21%; Oral sensitised: 14.24 ± 8.14%) compared to the SPF facility (IP sensitised: 2.98 ± 1.24%, $p < 0.0001$; Oral sensitised: 5.05 ± 1.87%. $p = 0.0002$).

Within each facility, genus level differences in the bacterial communities were observed between the allergy model groups and control group mice. A decrease in the relative abundance of bacteria from the genus *CAG-873* was observed (21.07 ± 1.36%) in IP sensitised mice compared to naïve animals (32.35 ± 8.87%, $p = 0.003$) maintained in the conventional facility. An increase in the relative abundance of bacteria from the genus *KNHs209* was observed in IP sensitised mice compared to IG sensitised mice (14.24 ± 8.14%, $p=0.0143$), while an increase in the relative abundance of *KNHs209* was observed in oral sensitised mice compared to naïve mice (4.83 ± 0.15%, $p < 0.0001$). In the SPF facility groups, an increase in the relative abundance of bacteria from the genus *Muribaculum* was observed in the IP sensitised and oral sensitised mice (IP sensitised: 26.18 ± 6.89%, $p < 0.0001$; Oral sensitised: 21.71 ± 6.60%, $p < 0.0001$) compared to naïve mice (9.09 ± 2.01%). Also, an increase in the relative abundance of bacteria from the genus *Ruminococcus* was observed in IP sensitised mice (7.02 ± 3.47%) compared to the naïve mice (0.27 ± 0.12%, $p = 0.0348$).

3.4 Discussion

Food allergy is serious health concern worldwide and a significant body of research to study food allergy mechanism is performed using pre-clinical models [33]. Mouse models are frequently used to elucidate the underlying immune mechanisms involved in allergic diseases, characterisation of novel allergenic proteins, pre-clinical development of therapeutic lead compounds, and more recently, in investigating the role of microbiota (skin, lung and gut) in allergic diseases[2,3,5,19]. The pathological and immunological outcomes of allergy mouse models have been shown to be impacted by the choice of mouse strain[34]. More recently mouse models of allergy have been used to investigate the role of specific gut microbial communities, isolated from allergic or healthy donors, to study their propensity to push the immune response towards sensitisation or immune tolerance[35,36]. It is important, in such instances, to understand the influence of external controllable factors that may play a role in these outcomes. Recently, a study by Parker et al. showed that the gut microbiome composition in either wild type or disease model (Hirschsprung disease) is influenced by the animal holding facility[13]. It is important to fully understand the impact the facility environment has on disease models to better address research questions in food allergy research, and to achieve reproducible outcomes. In this study, we have shown for the first time that mouse models of food allergy are significantly influenced by the housing conditions (specific pathogen free and conventional) and route of sensitisation used to establish the model.

A recent study performed on two different strains of mice has specifically shown that the choice of mice strain could potentially dictate the anaphylactic response[10]. Furthermore, strains of mice such as C3H/HeJ and BALB/c have been reported for their genetic susceptibility towards developing food allergy due to differential Th1/Th2 cytokine response[12]. Similarly, our study reports that different housing conditions (SPF and conventional) result in a significant difference in pathology in response to peanut sensitisation and challenge. The anaphylactic response, measured as hypothermic shock was prominent for both IP and oral sensitised allergy models in the conventional facility, but none in the SPF facility. Interestingly, this difference was not supported by the humoral response, including the IgE, IgG, or IgG2a responses, which was similar in

mice across both facilities. When cytokine responses were compared, the IP- and oral sensitised allergy groups in the conventional facility showed an upregulation of Th2 as well as Th1 cytokines, particularly in the IP sensitised group. Differential cytokine response in IP sensitised groups could indicate that the BALB/c mice used in our study have propensity towards T-cell based response as reported in previous studies[10,34,37]. In contrast, the allergy groups housed in the SPF facility showed a reduced cytokine response as compared to naïve mice, although the differences were not statistically significant. When total and degranulated mast cells were compared, there were no differences observed between groups across the two facilities. The similar number of mast cell presence could be the reason for undetected allergen specific IgE since no IgE bound mast cells were present therefore no significant degranulated mast cell population was detected in the gut tissue.

A comparative analysis was also made between two different routes of sensitisation: intraperitoneal and intragastric (oral sensitised). Mice sensitised intraperitoneally showed a significant temperature drop and increased IgE, IgG, and IgG1 antibody responses in comparison to oral sensitised mice. IP-sensitised mice showed an elevated Th2 cytokine response but without statistical support compared to oral sensitised mice. Previously, studies have reported that intraperitoneal allergen administration elicit better anaphylactic response, however, cellular, and humoral responses are dependent on strain of mice and food allergen[8,10,37,38]. Similarly, an example of strain differences was reported by Martin *et al.* stating C3H/HeOuJ strain demonstrated higher sIgE, sIgG1 and sIgG2a production with more severe clinical symptoms compared the BALB/c strain[10]. In addition, IP sensitised mice elicited a significantly higher degranulation response on challenge as compared to oral sensitised mice. Overall, in our study, IP-sensitised peanut allergic mice housed in the conventional facility showed a clear and measurable clinical and immunological response. The main difference between the facilities was observed mainly in the clinical response (hypothermic shock) upon allergen challenge.

To gain a better understanding of the observed clinical differences between the two types of mouse facilities, we performed a detailed comparative analysis of the gut microbiome. PERMANOVAs calculated based on Bray-Curtis Dissimilarity measure,

demonstrated a significant difference in bacterial community structure in mice based in the two different facilities as well as between the IP- and oral sensitised groups. However, when pairwise comparisons were made, significant differences were found in the SPF facility only, among sensitised mice as compared to naïve mice. Alpha diversity measures (Shannon index and Chao richness) showed that there was no difference in diversity between naïve mice and oral sensitised mice. Interestingly, a contrasting impact on the Shannon index and Chao richness were observed in IP sensitised mice in the SPF (decreasing effect) and conventional (increasing effect) facility.

Different relative abundances of specific bacterial communities were analysed at the genus level, to see whether they were associated with different allergy groups across the two facilities. The bacteria belonging to genus KNHs209 (Phylum: *Firmicute*s, Class: *Clostridia*) was more abundant in the conventional facility. Moreover, higher relative abundance was observed in IP sensitised and oral sensitised mice, where a clear allergic response to peanut challenge was observed. However, this finding was in contrast to studies which showed that bacterial community belonging to this phylum and class was either enriched in human subjects outgrowing milk allergy and suppressed an allergic response in mice[19] or involved in inducing colonic regulatory T cells[39]. In the SPF facility, higher relative abundance of bacteria belonging to the genus *Akkermansia* (phylum *Verrucomicrobia*) and *Muribaculum* (phylum *Bacteroidetes*) were observed as compared to the conventional facility. Bacterial populations from the phylum *Bacteroidetes* were shown to rebalance Th1/Th2 cytokine responses and induce regulatory T-cell development[19,34,35]. This may partially explain the lack of a measurable allergic response in mice housed in the SPF facility. In the current study, we could not directly correlate the presence of specific bacterial communities belonging to phyla or class, which are known to play a role in allergic sensitisation or tolerance, to the allergic phenotype obtained in the different allergy models in different facilities. Future studies focusing on faecal mass transplant (FMT) experiments could be conducted to generate conclusive data on the role of specific microbiome in allergy models.

In summary, our study demonstrated that mice housed in a conventional facility as compared to an SPF facility, elicited a clear clinical response indicating a significant drop in body temperature in a BALB/c mouse model of peanut allergy. Additionally, Intraperitoneal sensitisation gave a measurable humoral (IgE levels) and cellular response (mast cells) as compared to oral sensitisation in both SPF and conventional facility. In concurrence with previous study on disease models, we showed that the type of animal facility has an impact on gut microbial community in an allergy model. In contrast to the outcomes in Chapter-2, we showed for the first time that there is a direct effect of intraperitoneal route of sensitisation on the gut microbial diversity in sensitised mice. For such studies, it is important to consider the impact of route of sensitisation and mouse facility-type while designing and establishing an allergy mouse model.

3.5 Summary

Chapter-3 describes the investigation on the impact of animal housing facility and route of immunisation on the pathology, immune responses, and the gut microbial composition in a peanut allergy BALB/c mouse model. In Chapter-2, the mouse model for peanut allergy was unable to elicit a sustained clinical or immunological response in BALB/c mice. Based on the Chapter-2 findings and available literature we hypothesize that clinical symptoms and immune responses of a food allergy mouse model could be impacted by the type of housing facility and route of sensitisation.

Our results in Chapter-3 indicate a clear difference in the clinical symptoms and immunological responses across different housing facilities and route of sensitisation. Mice in the conventional facility had a stronger anaphylactic response upon allergen challenge compared to SPF facility, while no differences were observed in the humoral or cytokine responses. Intraperitoneal sensitisation elicited a stronger anaphylactic, antibody and cytokine response compared to intragastric route. The immune responses were accompanied by significant changes in the bacterial community structure in mice based on the facility or route of sensitisation. Intraperitoneal sensitisation had a contrasting opposite effect on bacterial alpha diversity in SPF (decreasing) and conventional (increasing) facilities. In conclusion, the outcomes of food allergy mouse models are impacted by housing conditions and route of sensitization and may be associated with alterations to the gut microbiota.

Another critical factor in Chapter-2 was to check the safety and efficacy of ulvan extract and purified ulvan polysaccharide. We did establish the safety profile for both extract and polysaccharide based on different dosage and using biochemical assay. Considering the important role of gut microbiota in food allergy, in Chapter-4 we investigated the impact of feeding purified ulvan polysaccharide and a carotenoid (astaxanthin) on the gut microbial community of mice. In addition to the dose-based pilot study and biochemical assay outcomes, microbiome data would help us in establishing the beneficial effects of ulvan polysaccharides isolated from green macroalgae *U. ohnoi* on gut microbiota.

3.6 References

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Chapter-4

Impact of algal polysaccharide ulvan and carotenoid astaxanthin on mice gut microbiome

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The algal polysaccharide ulvan and carotenoid astaxanthin both positively modulate gut microbiota in mice

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4.1 Introduction

The gut microbiota plays an essential role in human health and well-being. An imbalance or dysbiosis of the gut microbiota has been associated with several chronic and inflammatory non-communicable diseases such as obesity, type-2 diabetes, inflammatory bowel disease[1]. Current research is focused on investigating various natural supplements originating from either plants or marine algae to explore alternative potential therapeutic[2]. Furthermore, diet-based natural supplements have been reported to influence the gut microbiota[1,3]. Chapter 2 investigated the safety and efficacy of ulvan extract and a purified ulvan polysaccharide. However, a crucial aspect of the mouse models involving supplementation of natural compounds is their impact on the gut microbial community structure. The outcomes reported in chapter-3 dictates an evident influence of housing conditions on mice gut microbial community. Hence, in Chapter-4, the study aim was to investigate the impact of feeding only the purified ulvan extract and a well-researched carotenoid astaxanthin on the gut microbial community of mice without any pathology. A clear understanding of the factors influencing the mouse model will enhance our knowledge and refine the pre-clinical model design for further studies.

Diet and dietary fibres play a central role in maintaining gut homeostasis as bacterial populations use them to produce short-chain fatty acids (SCFAs) and other molecules, which interact with the intestinal mucosal barrier and assist in immune tolerance[3,4]. Different dietary supplements involving a range of macromolecules, such as polysaccharides, are efficacious in promoting the growth of beneficial bacteria to produce immune-boosting metabolites[3–5]. Dietary polysaccharides are mostly found in plant-based food products; however, untapped resources, such as both marine and freshwater algae, are increasingly gaining interest as a source of polysaccharides[2,6,7]. Algal polysaccharides from different origins are known for their immune-modulating properties and suppressing inflammatory responses[4,8].

Properties of a polysaccharide such as glycosidic linkages, molecular weight, monosaccharide composition, and sulphate content vary between polysaccharides and algal species[8,9]. However, unlike terrestrial plant polysaccharides, many algal polysaccharides are sulphated (e.g., fucoidan from brown, carrageenan, and agar from

red seaweed), contributing to their various structural properties and biological functions[10]. For example, *Ulva ohnoi* is a marine green macroalga rich in sulphated ulvan, composed of sugars (i.e., rhamnose and xylose) and other components such as different uronic acids[11,12]. As such, macro-and microalgal-derived polysaccharides have been explored for different purposes, such as alternative food products and nutraceuticals, or for their anti-inflammatory[10,13], antioxidant[14,15] and immunomodulatory activity[15,16].

Microalgae contain an abundance of various pigmented components, such as carotenoid xanthophylls and chlorophylls[17], that have also been reported to have beneficial bioactivities in health applications[18]. Astaxanthin (3,3′-dihydroxy-ß-carotene-4,4′-dione) is a carotenoid extracted from the freshwater microalga *Haematococcus pluvialis* but is also found as a major xanthophyll component in other microalgae and yeast[16,19]. It is also present in seafood, including shrimp, lobster, and salmon, after acquiring it through feeding on microorganisms that produce astaxanthin[16,20]. Astaxanthin is a secondary carotenoid easily distinguishable by its bright red colour and is structurally related to other carotenoids, such as β-carotene and lutein. Astaxanthin is a ketocarotenoid, meaning it contains hydroxyl and carbonyl functional groups making it a prime target for exploring the antioxidant properties in biomedical applications[20].

Ulvan and astaxanthin have been described for their beneficial bioactive properties; however, reports on their impact on the microbiota are scarce[21,22], particularly for purified extracts. We expect that the incorporation of ulvan and astaxanthin into the diet could have an overall impact on the gut microbiota. Therefore, this chapter investigated the effect of feeding the sulphated polysaccharide ulvan from *U. ohnoi* and the carotenoid astaxanthin from *H. pluvialis* on the murine gut microbiota using 16S rRNA gene sequencing. BALB/c mice were fed with either a control diet, ulvan, or astaxanthin for 28 days. Ulvan and the astaxanthin treatment changed the bacterial community structure compared to the naïve group of mice, increasing the relative abundance of classes *Bacteroidia*, *Bacilli*, *Clostridia*, and *Verrucomicrobia*. The study outcomes help us understand the potential impact of polysaccharides and carotenoids on the mouse gut microbiota, which may play an essential role in maintaining gut homeostasis, and subsequently, their therapeutic potential in inflammatory gut diseases.

4.2 Materials and methods

4.2.1 Animals

BALB/c, female, 6-8 weeks old mice (total n=15, n=5 per group) were obtained from the Australian Institute of Tropical Health and Medicine (AITHM) at James Cook University, Townsville, Australia. Mice were maintained on a 12 h light/dark cycle in individually ventilated cages (Tecniplast, NSW) in the SPF facility. This study and all protocols were carried out following the recommendations from an independent ethics committee for animal experimentation (Ethics ID: A2524).

4.2.2 Procurement of ulvan and astaxanthin

Ulva ohnoi was grown at scale in-house in a land-based aquaculture system at James Cook University as described previously[23]. The extraction of ulvan from *U. ohnoi* was performed by Marinova Pty Ltd. (Australia) using a proprietary mil aqueous process. Purification of the resulting extract was performed as described previously[10]. Astaxanthin from *H. pluvalis* was supplied by Pacific Biotechnologies Pty Ltd (Australia). Briefly, crude ulvan was dissolved in Type 1 water, vacuum filtered (Filtech, 453), and then diafiltered with five volumes of Type 1 water using an Äkta flux 6 system equipped with a 10,000 NMWC filter, UFP-10-E-4X2MA. Protein was removed from the retentate using anion exchange chromatography (AEC) (Äkta Pure 150L equipped with a single wavelength UV-detector at 280 nm). The column (XK 50/30 column, GE Healthcare Life Sciences) was equilibrated as follows; Type-1 water, 5 column volumes (CV); 2 M NaCl, 5 CV; Type 1 water, 5 CV, and the retentate was eluted using a stepwise gradient (0 M NaCl, 2 CV; 0–0.5 M NaCl, 2 CV; 0.5–1 M, 2 CV; 1–1.75 M NaCl, 3 CV; 1.75–2 M NaCl, 5 CV) at a flow rate of 20 ml min^{-1}). Fractions containing uronic acids (detected calorimetrically using the m-phenyl-phenol method with glucuronic acid as standard) were pooled and diafiltered to concentrate until the permeate conductivity was <5 µS cm^{-1}[10]. This purified ulvan was freeze-dried and then milled to a fine powder using mortar and pestle. Astaxanthin from *H. pluvalis* was supplied by Pacific Biotechnologies Pty Ltd (Australia).

4.2.3 Feeding regimen

6-8 weeks old mice were randomly separated into three groups: naïve, ulvan, and astaxanthin. Five mice per group were distributed and housed together during the experiment. The groups received purified ulvan extract (5 mg/mouse) and astaxanthin doses (1 mg/mouse) respectively via intragastric gavage every second day for 28 days [Fig 4.1]. Purified ulvan was prepared as 5 µg ulvan/200 ml PBS (25 µg ulvan/µl). The astaxanthin was procured as an emulsified solution in medium-chain triglyceride (MCT) oil. For our experiment, astaxanthin was prepared as 5 µl of emulsified astaxanthin solution in 195 µl of PBS with 1 mg astaxanthin (5 µg astaxanthin/µl) in solution. Drinking water and irradiated food pellets of soy-free rat and mouse reformulated diet (Specialty feeds, Australia) were fed to the mice *ad libitum*.

4.2.4 Sample collection

Mice were monitored briefly after feeding for any physical discomfort. Faecal samples were collected in DNase and RNase-free tubes on Day 0 and Day 28, two hours after feeding the ulvan and astaxanthin. After 28 days, the mice were sacrificed using CO_2 asphyxiation. Caecum samples with intact faecal matter were collected and snap-frozen in liquid nitrogen. All samples were stored at -80 ºC [Fig 4.1].

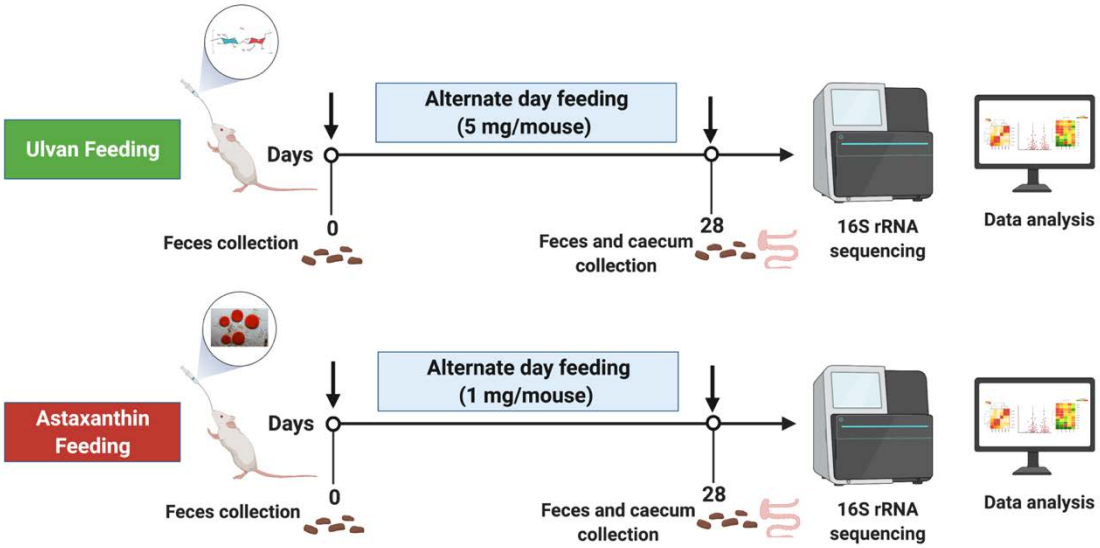

Fig 4.1: Feeding of ulvan and astaxanthin to BALB/c mice. Timeline depicting the feeding regimen of ulvan and astaxanthin on alternate days for 28 days. Faeces pellets were collected on Day 0 and Day 28 and caecum samples on Day 28 and analysed for the microbiome.

4.2.5 Microbiome community analysis

4.2.5.1 DNA extraction and 16S rRNA gene amplification and sequencing

Total DNA from the faecal and caecum samples (weight 200 mg) of mice was extracted using the DNeasy Powersoil kit following the manufacturer's instructions (Qiagen, Germany). The bacterial community composition of the samples was investigated by sequencing the V3-V4 hypervariable region of the 16S rRNA gene using the universal primers 341F & 785R as previously described[24]. Briefly, the amplification was performed for a final reaction volume of 50 µl per sample containing 2X Master Mix (Econotaq® PLUS GREEN, Lucigen), 10 µM of each primer, 20 ng/µl of template DNA. The cycling conditions for PCR included initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 40 s, and a final extension at 72°C for 7 min. The amplicons were quality checked by gel electrophoresis system and the then paired end sequenced (2 x 300 bp) on a MiSeq platform at the UNSW Ramaciotti Centre for Genomic as described in the User Guide (Illumina 2013).

4.2.5.2 Sequencing data analysis

The sequences of the V3-V4 region were analysed as described by Wemheuer and Wemheuer (2017)[25]. Briefly, quality-filtering and trimming of the paired-end reads were done using TRIMMOMATIC version 0.36[26]. USEARCH version 11.0.667[27] was used to merge read, and quality-filter them, excluding sequences with < 250 or > 550 nucleotides, in addition to sequences with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into amplicon sequence variants (ASV) using the USEARCH-UNOISE algorithm. The chimera detection was performed using UCHIME version 4.1[28] with the SILVA SSURef 132 NR database[29]. The ASV obtained were taxonomically classified by BLASTN[30] against the SILVA database. The ASV table was filtered to remove all non-bacterial, non-BLAST aligned, and singleton ASVs.

4.2.6 Community and statistical analysis

To assess the species richness in the samples, we generated rarefaction curves using the rarecurve function of the vegan package in R (version 3.5.3) as described previously[31]. For subsequent analysis, samples were normalized to 22,900 counts per sample. The alpha diversity in the sample population was calculated as a measure of observed species, ASV richness, and Shannon index in R using the rarefy function in the vegan package for community ecology analysis[32]. Briefly, alpha diversity is an indicator of diversity in a single sample measured using ASV richness. ASV richness is the number of ASVs with at least one read for each sample estimated using the Shannon index, i.e., an estimate of the diversity of the species in each sample[33]. A two-way ANOVA test in GraphPad Prism 8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups; a *p*-value <0.05 considered significant.

ASV tables were imported into PRIMER[34] for multivariate analysis of microbial communities to compare the community structure (i.e., relative abundance data). Bray-Curtis similarity coefficients were calculated using square-root transformed ASV abundances, and the resulting similarity matrix was visualized using non-metric, multidimensional scaling (nMDS). Permutational multivariate analysis of variance (PERMANOVA)[35] with 9999 random mutations was used to test the effect of sample type, treatment, and time on microbial communities in mouse faecal samples. 'Sample type' ("faecal" or "caecum") was a fixed factor, 'Treatment' ("naïve", "astaxanthin" and "ulvan") was a fixed factor and 'Time' ("Day 0" and "28") was a fixed factor.

4.3 Results

4.3.1 Bacterial community recovery from samples

We used a 16S rRNA gene-based analysis to assess bacterial communities from mouse faecal and caecum samples. After quality filtering, there were a total of 1,625,935 sequencing reads clustered into 341 ASVs. Rarefaction analysis and an

average good's coverage of 99.95% ± 0.04% indicated that the given sequencing effort recovered the majority of the bacterial diversity in the samples [Fig. 4.2].

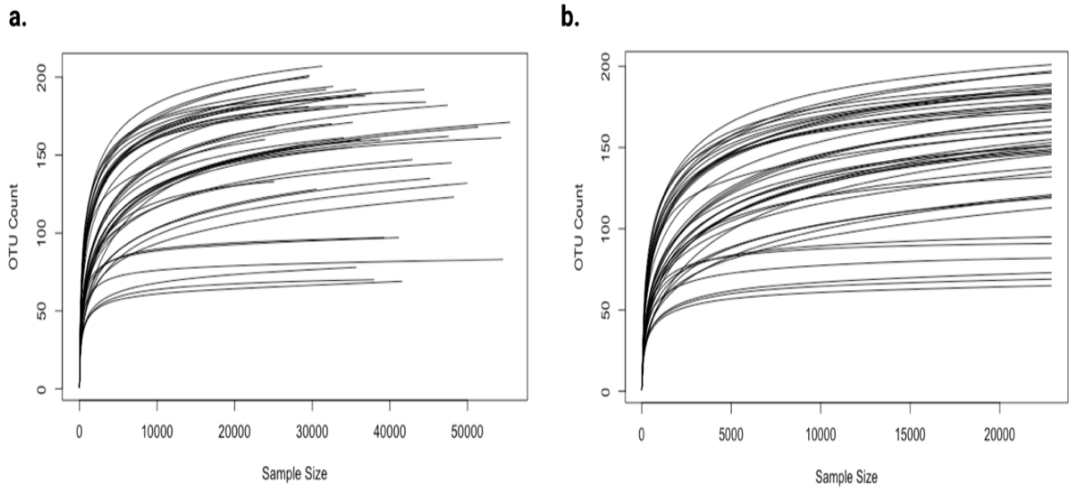

Fig 4.2: Total operational taxonomic unit (OTU) count before and after rarefaction analysis. Rarefaction curves (a) before and (b) after normalization (22,900 sequences).

4.3.2 Diversity and richness of microbiota in ulvan and astaxanthin fed groups

There was no statistical support for differences in diversity or richness between the faecal and caecum samples on day 28 ($p > 0.05$) [Fig 4.3a, 4.3c]. There was an increase in diversity for day 28 samples compared to day 0, which was more pronounced for the 'ulvan' treatment ($p = 0.0114$) [Fig. 4.3b]. There was no statistical support for differences in richness between the treatments on days 0 and 28 ($p > 0.05$) [Fig. 4.3d].

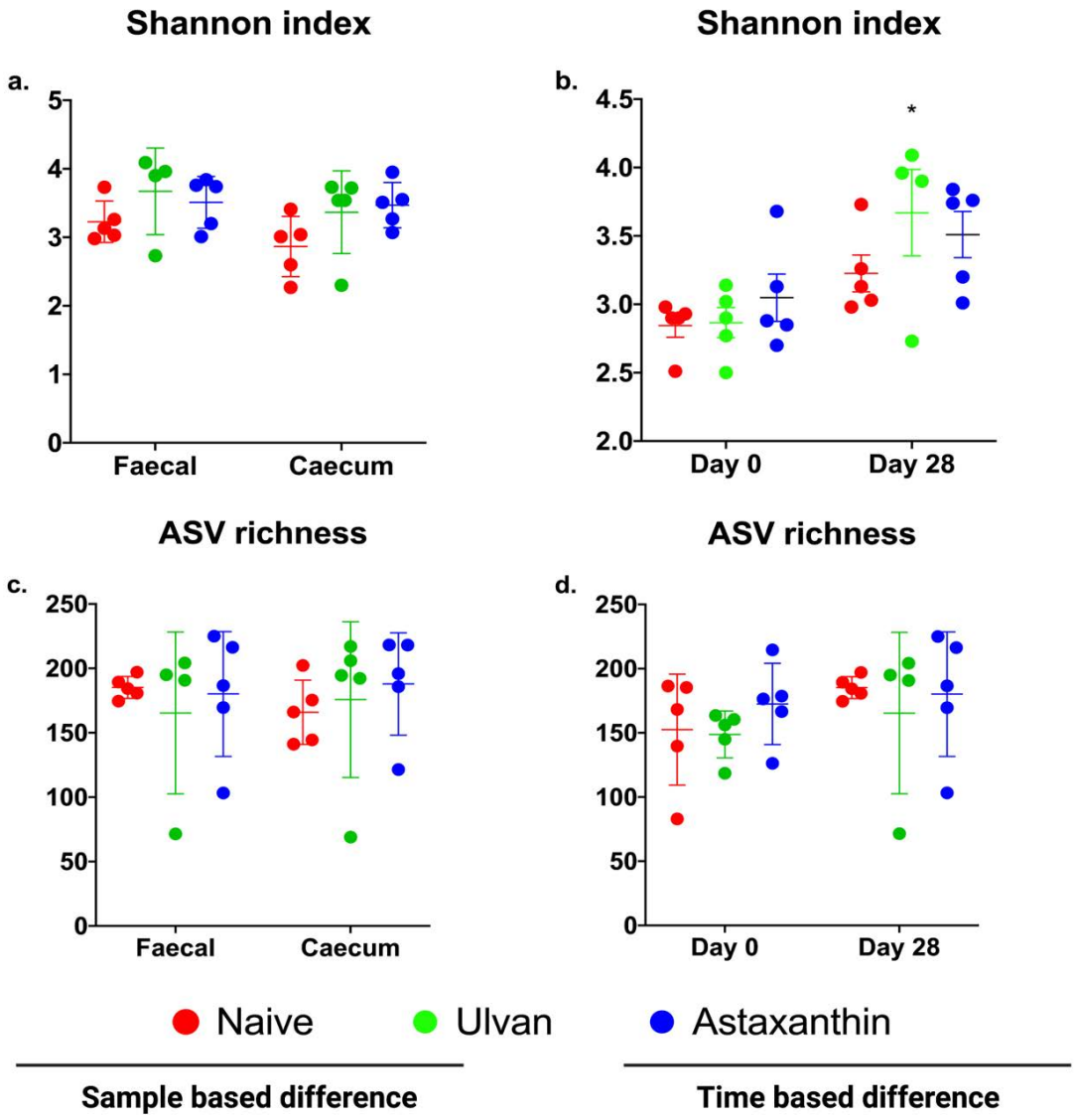

Fig 4.3: Differences in the diversity and richness between the fecal and caecum samples based on the type of sample and time (Day 0 and 28) as shown using Shannon index and ASV richness. Shannon diversity index (a, b) and ASV richness (c, d) data based on sample and time difference are presented as mean ± SEM. Tukey's pairwise comparisons test was used to determine the significance between the different groups (Day 0 & 28) with a *p*-value <0.05.

4.3.3 Algal polysaccharide feeding affect bacterial community structure and relative abundance of bacterial diversity

An effect of 'time' was observed on the overall bacterial community structure from faecal samples collected on days 0 and 28 [Fig. 4.4a, Table 4.1; PERMANOVA: p = 0.0422]. In addition, an effect of 'treatment' was observed on the overall bacterial

community structure based on Bray-Curtis dissimilarity irrespective of sample type (i.e., faecal or caecum) on day 28 [Fig. 4.4b, Table 4.2; PERMANOVA: $p = 0.0121$].

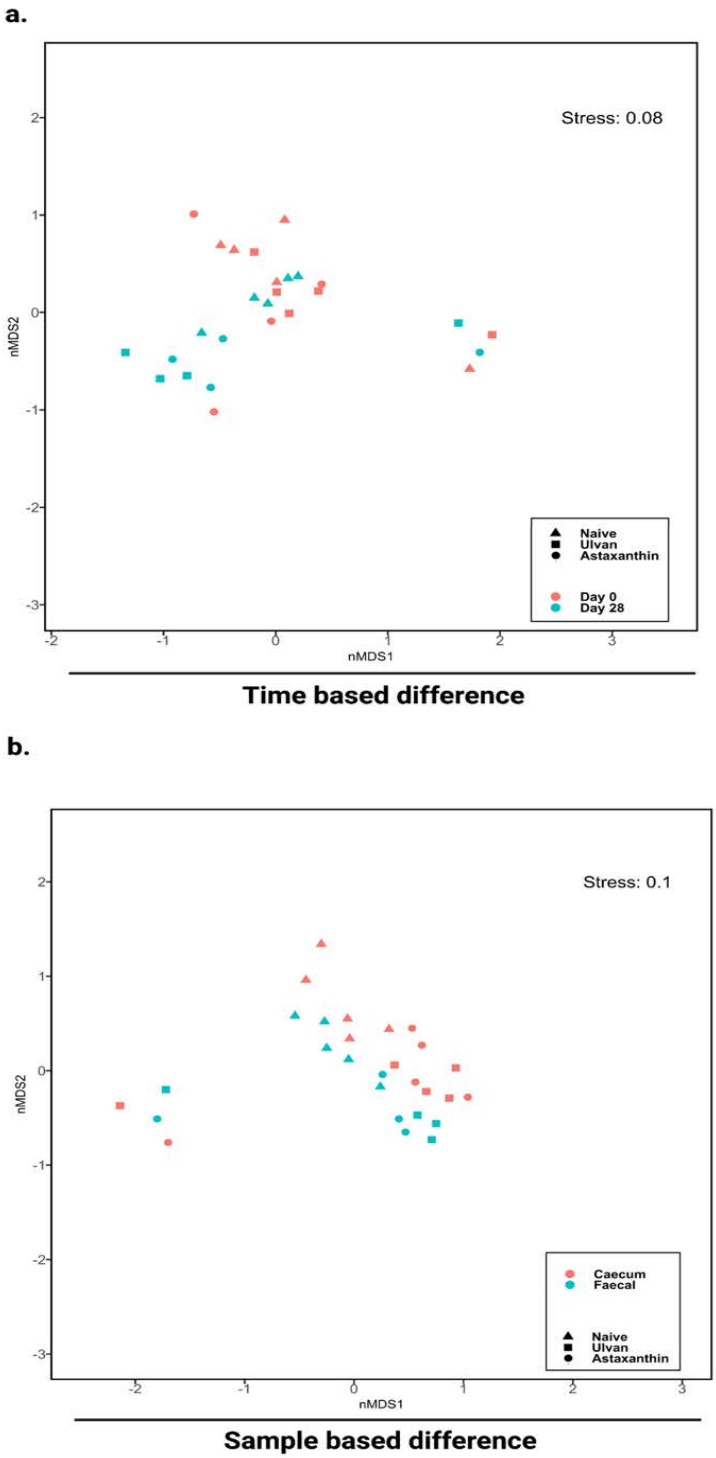

Fig 4.4: Multidimensional scaling (MDS) plot of bacterial community structure differences upon ulvan and astaxanthin feeding, based on sample type, i.e., time-based (a) and faecal and caecum samples (b), at Day 0 and Day 28.

There was statistical support for differences between naïve samples and samples supplemented with astaxanthin or ulvan [Table 4.3; PERMANOVA: p = 0.0039, p = 0.0037; respectively], indicating an effect of algal extract feeding on the bacterial community structure.

Table 4.1: PERMANOVAs based on Bray-Curtis (BC) similarity measure for square-root transformed abundances of all mice faecal samples collected on day 28. p-values were calculated using 9,999 permutations under a residual model.

Source	df	SS	MS	Pseudo-F	p (perm)	Unique perms
Treatment	2	1476.6	738.28	1.1211	0.347	9918
Time	**1**	**1599**	**1599**	**2.4283**	**0.0422***	**9938**
Treatment X Time	2	1203.6	601.81	0.91389	0.4955	9932
Res	21	13829	658.52			
Total	26	18002				

Bold and * indicates statistically significant values (at alpha = 0.05).

Table 4.2: PERMANOVAs based on Bray-Curtis (BC) similarity measure for square-root transformed abundances of all mice faecal and caecum samples collected on day 28. p-values were calculated using 9,999 permutations under a residual model.

Source	df	SS	MS	Pseudo-F	p (perm)	Unique perms
Sample	1	1218.3	1218.3	1.8142	0.1204	9945
Treatment	**2**	**3657**	**1828.5**	**2.7229**	**0.0121***	**9937**
Sample X Treatment	2	307.19	153.6	0.22873	0.9993	9927
Res	22	14773	671.52			
Total	27	19938				

Bold and * indicates statistically significant values (at alpha = 0.05).

Table 4.3: Pairwise comparison tests between groups.

Groups	t	p (perm)	Unique perms
Astaxanthin, Naïve	**1.9029**	**0.0039***	**9939**
Astaxanthin, Ulvan	0.75135	0.6475	9944
Naïve, Ulvan	**2.1882**	**0.0037***	**9936**

Bold values marked as * are statistically significant based on $p > 0.05$.

4.3.4 Taxonomic structure of the bacterial communities after ulvan and astaxanthin feeding

The most abundant bacterial classes found in the faecal samples for different treatment groups on days 0 and 28 belonged to the classes *Bacteroidia*, *Bacilli*, *Clostridia,* and *Verrucomicrobia* [Fig 4.5a]. Other bacterial classes were present at lower relative sequence abundance levels in some (but not all) faecal samples both on days 0 and 28 [Fig 4.5b]. A higher relative abundance of bacteria from the class *Verrucomicrobiae* was observed in naïve samples (day 0: 26.45% ± 4.40%; day 28: 21.87% ± 2.86%) compared to astaxanthin-fed samples (day 0: 13.97% ± 8.44%, p = 0.0331; day 28: 10.93% ± 9.38%, p = 0.0293), while a lower relative abundance of bacteria from the class *Clostridia* was observed in the naïve samples (28.28% ± 1.91%) compared to ulvan-fed samples (39.20% ± 13.48%, p = 0.0425) on day 28 [Fig 4.5a].

The bacterial families *Muribaculaceae* (class *Bacteriodia*), *Lachnospiraceae* (class *Clostridia*), *Lactobacillaeceae* (class *Bacilli*), *Ruminococcaceae* (class *Clostridia*), and *Akkermansiacaeae* (class *Verrucomicrobia*) were most abundant in the faecal samples for different treatment groups on days 0 and 28 [Fig. 4.5b]. A lower relative abundance of bacteria from the family *Akkermansiaceae* was observed in astaxanthin-fed samples (day 0: 13.97% ± 8.44%, p = 0.0331; day 28: 10.93% ± 9.38%, p = 0.0293) compared to naïve samples (day 0: 26.45% ± 4.40%; day 28: 21.87% ± 2.86%), while higher relative abundances of bacteria from the family *Lachnospiraceae* was observed in the ulvan-fed samples (33.20% ± 10.31%, p = 0.0002) on day 28 compared to naïve samples (12.87% ± 7.86%) [Fig 4.5a]. A higher relative abundance of bacteria from the family *Lachnospiraceae* was observed in the ulvan-fed samples on day 0 (9.07% ± 3.63%) compared to day 28 (33.20% ± 10.31%, p < 0.0001). Additionally, on day 28 an increase in the relative abundance of *Lachnospiraceae* was also observed in astaxanthin-fed (12.31% ± 8.30% to 26.29% ± 10.42%) samples. In comparison, the lower relative abundance of bacteria from the family *Ruminococcaceae* was observed in the astaxanthin-fed samples on day 0 (21.42% ± 13.16%) compared to day 28 (6.10% ± 5.11%, p = 0.0146) [Fig 4.5b].

Faecal samples for different treatment groups on days 0 and 28 were abundant in the genus *Akkermansia* and the uncharacterized genera *Muribaculaceae A2*, *Lachnospiraceae NK4A136 group*, *Lachnospiraceae UCG-008*, *uncultured*

Lachnospiraceae, *Ruminococcaceae UCG-003*, *Ruminococcaceae UCG-014,* and *uncultured Ruminococcaceae*. A lower relative abundance of bacteria from the genus *Akkermansia* was observed in the astaxanthin-fed samples (13.97% ± 8.44%) compared to naïve and ulvan-fed samples (naïve: 26.45% ± 4.40%, p = 0.001; ulvan: 23.53% ± 7.11%, p = 0.0155) on day 0, as well as a decrease in the astaxanthin-fed samples (10.93% ± 9.38%, p = 0.0034) compared to the naïve samples (21.87% ± 2.86%) on day 28 [Fig 4.5c].

A higher relative abundance of bacteria from the genus *Ruminococcaceae UCG-014* was observed in the naïve samples (11.52% ± 7.13%) compared to the astaxanthin (1.61% ± 1.25%, p = 0.0064) and ulvan-fed samples (0.15% ± 0.1%, p = 0.0042) on day 28 [Fig 4.5c]. Also, a higher relative abundance of bacteria from the genus *Ruminococcaceae UCG-014* was observed in the astaxanthin and ulvan-fed samples on day 0 (astaxanthin: 16.07% ± 13.12; ulvan: 14.69% ± 8.30) compared to day 28 (astaxanthin: 1.61% ± 1.25%, p = 0.0002; ulvan: 0.15% ± 0.1%, p = 0.0008) [Fig 4.5c].

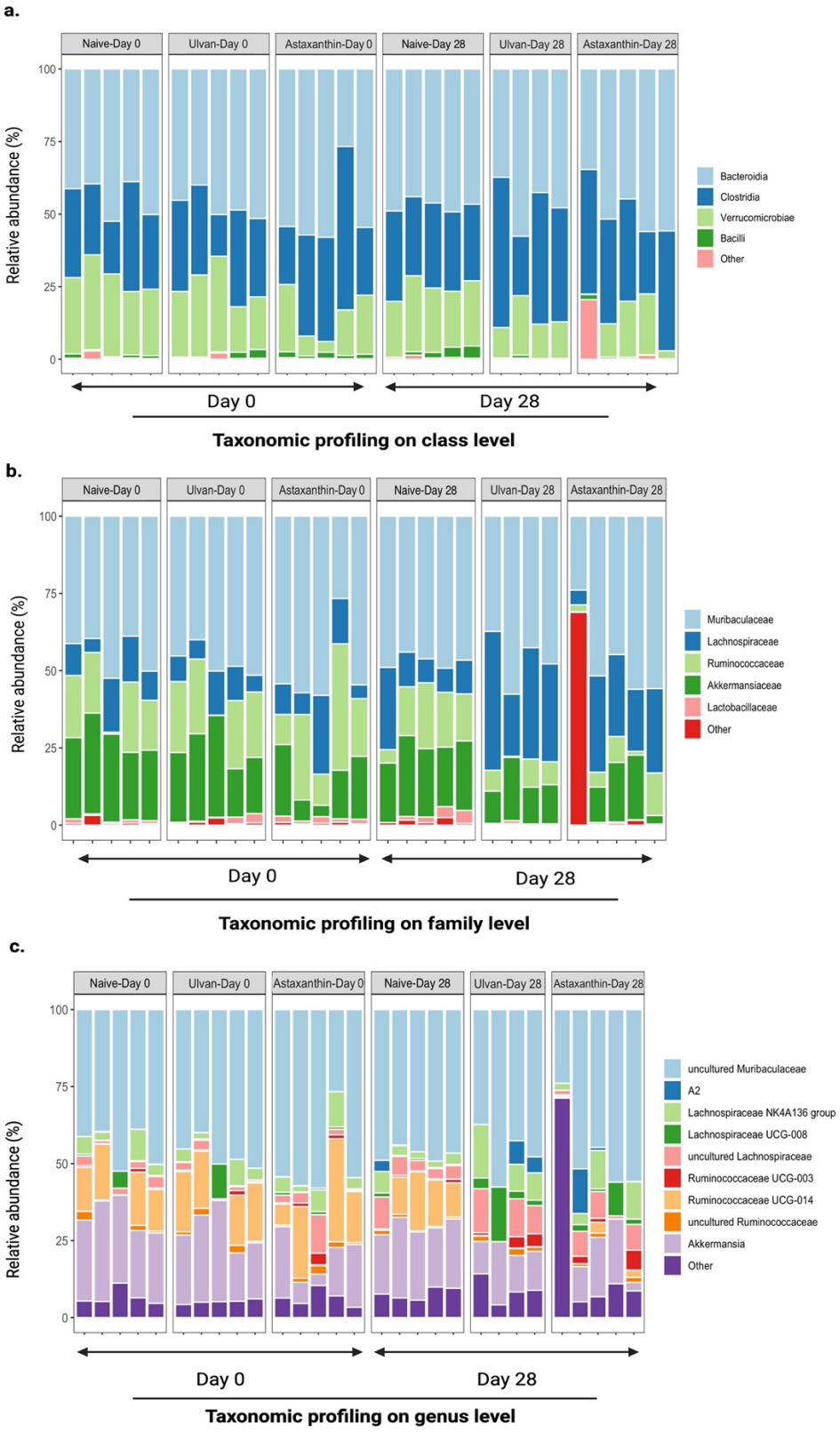

Fig 4.5: Taxonomic profiles of bacterial communities at class (a), family (b), and genus (c) level of all faecal samples collected from ulvan and astaxanthin-fed mice from Day 0 and Day 28.

4.4 Discussion

Ulvan and astaxanthin supplementation in mice changes the structure of gut microflora compared with naïve control mice, dominated by bacterial populations in the faecal samples belonging to classes *Bacteroidia*, *Bacilli*, *Clostridia*, and *Verrucomicrobia*, and their role has been attributed as a probiotic class of bacteria that can help in maintaining the intestinal barrier in mice and rats. Most of these microbial classes of bacteria have been reported previously for fermenting the polysaccharide into short-chain fatty acids (SCFA's) and other metabolites in the gut[1,18]. Natural polysaccharides and carotenoids originating from algae have been widely studied for their roles in immune protection[36,37]. However, the effects of algal polysaccharides and carotenoids on gut microbiota regulation have not been extensively studied.

This study detected an increase in gut microbiota richness with time in our polysaccharide-fed samples compared to naïve samples. Metabolites produced after the breakdown of such polysaccharides may be a source of nutrients for other beneficial bacteria, thus maintaining the gut homeostasis[5]. Polysaccharides are considered important regulators of microecology in the gut, directly affecting the selective colonization of intestinal flora[1]. Furthermore, the family *Lachnospiraceae* (*Clostridia*) was observed in faecal samples, which possess some beneficial xylan/fibre-degrading bacteria, such as *Eubacterium halli*, that have been reported for their butyrate-producing properties and facilitating the degradation of indigestible dietary fiber[38]. Previous studies on mice have shown that the loss of bacteria from the *Lachnospiraceae* family is linked with increased incidences of inflammatory bowel diseases and chronic gastrointestinal tract infections[39]. Also, a reduced abundance of *Lachnospiraceae* in an *in vitro* culture of patients with ulcerative colitis was associated with the relapse of disease condition due to low butyrogenesis, leading to ulcerative colitis recurrence[40].

In our study, we observed a reduction in the family *Ruminococcaceae* (*Clostridia*) in ulvan-fed mice with time, which is associated with a healthy gut and previously shown to be upregulated in mice after treatment with polysaccharides extracted from seaweed *Porphyra haitanensis* (Rhodophyta) and *Ulva prolifera* (Chlorophyta)[41]. The reduction of the *Ruminococcaceae* family in our study could be an indicator of the inaccessibility

of carbohydrate-binding modules provided by purified extract of ulvan to the gut bacteria[1,41]. Some gut bacterial species are more specific than others regarding substrate specificity and degrade different amounts of glycans based on the available substrate types[1].

The increase in the relative abundance of bacterial populations at the family level in the ulvan and astaxanthin fed groups corroborates the previous reports that dietary feeding of algal polysaccharides or carotenoids could also carry therapeutic value as prebiotic supplements[22,42–44]. Various diet regimens have been shown to decrease the impact of the opportunistic bacterial population, i.e., by increasing the population of beneficial bacteria and suppressing inflammatory responses in the gut[45]. In accordance, polysaccharides from different origins have been reported for several bioactive properties, including the modulation of the bacterial population in the gut[1]. Polysaccharides isolated from *Pleurotus eryngii*, an edible mushroom species, have been reported to increase the families of commensal bacterial populations, namely members of the families *Lactobacillaceae, Porphyromonadaceae*, *Bacteroidaceae*, and *Rikenellaceae*[46]. In another study, Tang et al. reported that diluted and concentrated alkali-soluble polysaccharides from purple sweet potato [*Ipomoea batatas* (L.) Lam] increased the population of *Bacteroidetes*, *Lachnospiraceae*, *Ruminococcaceae*, and *Oscillospira* that produce SCFA's such as butyric acid, acetic acid, and propionic acid in the mouse gut[43]. Additionally, similar studies have reported that digestion of polysaccharides in the gut can regulate the gut bacterial population and modulate the gut metabolite production to benefit overall gut health[42,47,48].

Additionally, the role of the gut bacterial population in the fermentation of the natural compounds also plays a significant role in maintaining gut homeostasis through SCFA's and other metabolite production[1]. Bobin-Dubigeon et al. reported lower degradation rates and fermentation of ulvan compared to individual sugars in an *in vitro* experiment using human faecal microbiota. However, the sugar constituents (rhamnose, ulvanobiouronate, and glucuronate) were found to be highly fermentable, suggesting that sugars are readily taken up after digestion by the gut microbes[49,50]. Sugar constituents in ulvan have been reported elsewhere for their efficacy in modulating

immune responses[8]. Interestingly, a similar study on ulvan fermentation using the human faecal microbiota reported an increased abundance of *Bacteroides*, *Lactobacillus,* and *Bifidobacterium* after 12 h of culturing[44]. Although ulvan from various other Ulva species have been reported for their efficacy in modulating the immune response[2,44,51], our study is the first to elucidate the effect of ulvan from *U. ohnoi* on mouse gut microbiota.

Our study supports the beneficial effect of astaxanthin as demonstrated by an increase in *Lachnospiraceae* families, whose members can ferment dietary substrates to beneficial SCFAs such as butyrate[40]. Based on an increased relative abundance of *Lachnospiraceae*, our findings suggest that astaxanthin can be investigated further for its potential as an immunomodulator and could improve gut health. In a recent clinical study of concordant and discordant cohort of identical twins, the non-allergic cohort were reported to have a higher abundance of the bacterial class *Clostridia,* especially *Lachnospiraceae* or *Ruminococcaceae* in their faecal samples as compared with the allergic cohort of twins[52]. The authors suggest these results indicate a link between the lack of *Lachnospiraceae* or *Ruminococcaceae* and increased allergic sensitization in the group[52]. Furthermore, in previous studies, members of the class *Clostridia* have also been reported to protect peanut sensitized mice[53]. Additionally, ulvan and astaxanthin have been shown in our study to increase the relative abundance of *Firmicutes* that belongs to class *Clostridia*; this provides us a proof of concept to study that these polysaccharides may have beneficial effects as prebiotics.

In immunological aspects, astaxanthin is a potent antioxidant and anti-inflammatory compound that has been widely studied and is used commercially as a nutraceutical[16]. Astaxanthin has a unique structure having both hydroxyl and keto groups attached, providing lipophilic and hydrophilic properties. These properties allow the compound access through the cell membrane, and it can also cross the blood-brain barrier and exert potential effects[16,54]. Astaxanthin, a natural added supplement in food, has been demonstrated to be beneficial *in vitro* and *in vivo* systems against various diseases, such as cancer, obesity, and diabetes[54]. Astaxanthin promotes M2 polarization and macrophage activation in case of inflammation and has been reported for potent anti-inflammatory properties such as inhibiting pro-inflammatory cytokines via NOD

signalling pathways in the case of atopic dermatitis[55–58]. The innate lymphocyte cells (ILCs) are immune cells produced in the intestinal barrier system[59]. Astaxanthin may also assist in ILCs differentiation upon digestion, especially ILC1, which are lymphocytes very similar to Th1 cells and can express pro-inflammatory cytokines such as TNF-α and IFN-γ upon foreign pathogen interactions in the gut[60]. An *in vitro* study also indicated the role of astaxanthin as a potential anti-allergic compound possessing anti-histamines-like activity to inhibit pathological immune activation of T-lymphocytes in case of allergic rhinitis and seasonal allergies[61].

Astaxanthin has recently gained attention for its role in maintaining immune homeostasis through gut health[22,62]. A recent study in C57BL/6J mice showed the potential impact of astaxanthin on the cecal gut microbial diversity[22]. This study suggested that the administration of astaxanthin alters the microbial signatures and regulates metabolic homeostasis in a gender-specific manner[22]. Its role in sugar metabolism in a high-fat diet mouse model by boosting the carbohydrate metabolism, lowering the blood glucose level and insulin resistance through maintaining intestinal integrity provided a clue for the potential use of this natural polysaccharide in metabolic disorders[63].

In summary, natural algal extracts such as ulvan and astaxanthin assist the propagation of beneficial microbial populations such as *Bacteroidia*, *Bacilli*, *Clostridia*, and *Verrucomicrobia* in the gut. Furthermore, ulvan and astaxanthin, as described in this chapter, can improve the relative abundance of the commensal bacterial population in the mouse gut and hence can be further explored as potential prebiotic supplements in future studies.

4.5 Summary

Chapter-4 describes the investigation on the impact of feeding the algal polysaccharide ulvan and carotenoid astaxanthin on the mouse gut microbiome. In Chapter-2, a pilot study on the safety and efficacy of ulvan extract and purified ulvan polysaccharide was conducted and found purified ulvan to be a safter option compared to extract. Based on the findings of Chapter-2 and 3 it was hypothesised that apart from the immunological and pathological differences induced due to housing, the feeding of polysaccharide to mice will likely introduce changes in gut microbiota. Therefore, Chapter-4 investigated the effects of purified ulvan polysaccharide and the carotenoid astaxanthin on normal mice to elucidate the changes in the gut microbiota due to feeding of these extracted natural compounds.

The results in Chapter-4 report a clear difference in the intestinal microbial community structure with a significant increase in the bacterial classes *Bacteroidia*, *Bacilli*, *Clostridia*, and *Verrucomicrobia* after feeding the mice with ulvan and astaxanthin. Duration of the treatments (28 days) had a more substantial effect on the bacterial community structure than the type of treatment (ulvan or astaxanthin). Our findings highlight that ulvan and astaxanthin could mediate aspects of host-microbe symbiosis in the gut, and if incorporated into the diet, these could assist positively in improving disease conditions associated with gut health. In conclusion, the feeding of ulvan and astaxanthin impact on the commensal bacterial population that can be studied further in disease oriented pre-clinical models.

In Chapter-2 the impact of ulvans on allergic immune responses was evaluated in a murine model of peanut allergy. While the sensitisation of mice to allergenic proteins from peanuts was not very affective, the expansion of peanut allergy to possible treatment of other nut allergies could be further evaluated. Cross-reactivity of peanut allergy with other nut allergen has been described, but particularly the potential cross-reactivity to the native Australian macadamia nut has not been well investigated. In Chapter-5 three purified allergens from macadamia nut were characterised and IgE reactivity was evaluated with a cohort of Australian individuals with multiple peanut/tree nut allergy.

4.6 References

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Chapter-5

Macadamia nut extract and purified allergen reactivity in peanut-allergic individuals

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Identification of vicilin, legumin and antimicrobial peptide 2a as macadamia nut allergens

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5.1 Introduction

Tree nut allergy is affecting approximately 4.9% of the general population worldwide[1]. However, peanut is still the most common nut allergen source, resulting in nearly 6-8% of allergy incidences in children[2–4]. In Chapter-5, the study aim was to investigate the potential of macadamia nuts and their purified proteins to mount cross-reactive IgE binding to a patient cohort of clinically confirmed peanut or tree nut allergic patients. The patient cohort comprised clinically confirmed peanut-allergic patients divided into three groups based on their skin-prick test evaluations to macadamia nut. Peanut and macadamia nuts share similarities in their protein composition, with 2S albumin and non-specific lipid transfer proteins (nsLTP) being the primary sources of allergens shared amongst them[5]. However, the literature on cross-reactivity of macadamia nut proteins with peanut proteins is yet not established. Therefore, Chapter-5 discusses the *in vitro* IgE reactivity of macadamia nut extract and its purified proteins in peanut-allergic patient serum. Based on potential cross-reactivity between tree-nut and peanut allergens, the development of immunotherapeutic for peanut allergy, as discussed in Chapter-2, might also be beneficial for individuals with tree-nut allergy.

Botanically, the tree nut is a dry fruit with an inedible hard shell and a seed; however, in broad terms, “Tree Nut” describes any nut coming from a tree. Though they are of high nutritional value and are part of a healthy diet, they can also be a potent source of food allergic reactions that trigger an IgE-mediated hypersensitivity response[1,6]. Among all known varieties of tree nuts, almond, walnut, pistachio, pecan, cashew, Brazil nut, pine nut, hazelnut and macadamia have been responsible for most allergies to tree nuts[7]. Amongst all, the global production of macadamia nuts and its consumption has expanded globally due to its popularity as a major ingredient in various bakery food items[8]. According to Australia’s epidemiological food allergy trends, it is likely that cases of macadamia allergy will increase in the future from the current status of 0.2%, especially among children and young adolescents[9,10]. In western countries such as the US, self-reported macadamia nut allergy prevalence is still low at 0.1%[11]. However, more macadamia nut allergy cases are observed among the European population, ranging between 10-17% in different geographical regions[12]. Considering the increased consumption of macadamia nuts in modern cuisine such as confectionery items, cereals and ice-creams, macadamia-nut allergy cases are predicted to increase in the coming

years[13]. Also, it is becoming a popular ingredient in products such as cold-pressed macadamia oil that is used extensively for baked goods, which may pose a threat to patients with existing nut allergy, depending on the method of processing[8,14].

Macadamia nut belongs to the genus of nine species of flowering plants in the family *Proteaceae*. There are only two species, Macadamia *tetraphylla* and *Macadamia intergrifolia,* that are considered commercially important[15]. The trees from other species such as *M. whelanii* and *M. ternifolia* tend to possess poisonous and/or inedible nuts due to the presence of cyanogenic glycosides, a toxic compound[15]. All these macadamia species are native to eastern Australia and bloom throughout tropical Australia. These nuts are also called Australian nuts, bopple nuts, bush nuts, and Queensland nuts in general[15].

As for many other tree nuts, allergic reactions to macadamia nut can range from mild oral symptoms to potentially life-threatening anaphylaxis[9,13,16–19] The clinical symptoms associated with macadamia induced allergies are highly varied and heterogeneous. It could be a typical oral allergy syndrome (OAS), causing discomfort to the oropharynx and severe life-threatening anaphylaxis[6,20]. A survey evaluating the allergies to nuts in America showed 115 allergic reactions to nuts, where 4% of responses are due to macadamia nuts (5/20) presented with moderate to severe systemic symptoms[6]. In young toddlers, anaphylaxis immediately following the ingestion of macadamia nut has been reported[20]. In a few nut sorters, occupational dermatitis on contact with macadamia nut shells at a macadamia nut processing plant was also described. However, the nature of the allergen in most macadamia cases is still unclear[21]. Reports of a 17.4 KDa protein appear to be the major allergen and is present in raw and roasted extracts[18].

Similar to other nut allergies, a macadamia allergy diagnosis generally relies upon a compelling history combined with a positive serum IgE and skin prick test (SPT). Also, in unclear cases, an oral food challenge is prescribed. In most cases, still, the physicians rely upon quantification of specific IgE (sIgE) to confirm their clinical suspicion in case of macadamia induced allergy. In some instances, it has been shown that quantification of sIgE for macadamia can produce false-positive outcomes showing cross-reactivity to hazelnut[22]. Considering the botanical viewpoint, the macadamia and hazelnut are

distantly related species; however still shows antigen homology in many cases[19]. Nevertheless, the clinical landscapes of macadamia caused allergy in childhood and its cross-reactivity with other nuts except hazelnut remain understudied. In recent years component-resolved diagnostics, or molecular diagnosis using allergen analysis, are becoming more utilised, accurate and advanced means of diagnosing the tree nuts allergies[23]. In patients with a suspected tree nut allergy, the molecular diagnosis approach quantifying the sIgE levels has proven beneficial in elucidating distinct sensitisation phenotypes. It can potentially replace the need for extract-based SPTs[24]. However, the investigations to find the culprit allergen in the case of macadamia allergy are ongoing, and the present knowledge is insufficient for improved diagnosis.

The main protein families involved in macadamia nut allergy include 2S albumins, 7S globulins (vicilins), 11S globulins (legumins) and non-specific lipid transfer proteins (nsLTP)[1]. Recently, vicilin, a seed storage protein constituent of the macadamia nuts, showed promising IgE sensitisation. In a recent study, Ehlers *et al.* showed vicilin-like antimicrobial peptides (VLAP) 2–1, 2–2 and 2–3 as novel allergens and has been suggested as a potential indicator for systemic reactions to macadamia nuts[16]. The vicilin-like 2-3 antimicrobial peptides are the most abundant protein found in extracts of macadamia nut. However, they display high sequence similarity with the N-terminal part of the walnut allergen Jug r 2. This has implied a potential cross-reactivity and false-positive detection for macadamia allergy[25]. Due to limited literature on macadamia allergens involved in cross-reactivity with other allergenic foods, the significance of individual components for molecular diagnosis remains largely unknown.

The present study describes the characterisation of natural and recombinant IgE-binding macadamia nut proteins and macadamia extract that can be used to form a panel of allergens. Our approach implies the use of various immunological methods to increase the adequacy of our identified allergens for molecular diagnosis in the future. Our study showed protein extracts from macadamia with several IgE-binding components ranging from low to high molecular weight (10–70 kDa). Together, the isolated IgE-binding proteins form a panel of macadamia allergens which may be helpful in future studies to improve the accuracy of macadamia allergy diagnosis.

5.2 Materials and methods

5.2.1 Serum samples

The Australian HealthNuts study is a comprehensive population-based study of food allergy consisting of a cohort of 5276 children enrolled at age 1 and followed up to 6 years of age[26,27]. For our study, a subset of patients recruited at 4–6 years with macadamia nut outcomes was investigated [Table 5.1].

Table 5.1: Characteristics of macadamia nut allergic and tolerant patients: clinical symptoms and IgE sensitisation as determined by SPT.

Patients no.	Age (y)/sex	Macadamia allergy	Symptoms to macadamia (reported at 6y)	Macadamia SPT wheal at 6 y (Ø mm)	Allergy to other foods at 4-6 y
Patients with macadamia nut allergy (MA 1–8)					
MA 1	6.1/M	yes	Current avoidance, last reaction >12months	8.0	Peanut, Cashew, Hazelnut, Pistachio
MA 2	6.3/M	yes	Current avoidance, last reaction >12months	15.0	Hazelnut, Walnut
MA 3	1.1/F	yes	Vomiting, hives	25.0	Hazelnut, Brazil nut, Pistachio
MA 4	4.4/M	yes	Current avoidance, last reaction >12months	10.0	Cashew, Pistachio
MA 5	6.1/F	yes	Current avoidance, last reaction >12months	11.0	Peanut, Cashew, Hazelnut, Brazil nut
MA 6	4.2/M	yes	Current avoidance, last reaction >12months	10.0	Peanut
MA 7	1.2/F	yes	Facial swelling, hives	10.5	No other food allergies
MA 8	1/1/M	yes	Current avoidance, last reaction >12months	10.0	Pecan, Walnut
Patients tolerant to macadamia with SPT 3-7 mm (MT 1-12)					
MT 1	6.1/F	no	AS	2.5	Peanut
MT 2	4.2/M	no	AS	5.0	Peanut, Cashew, Pistachio
MT 3	6.2/M	no	AS	4.0	Peanut
MT 4	4.1/F	no	AS	3.0	Cashew, Pistachio
MT 5	6.2/M	no	AS	4.5	Peanut
MT 6	1.1/M	no	AS	4.0	Peanut, Cashew, Pistachio, Walnut
MT 7	4.1/M	no	AS	3.0	Pecan, Sesame
MT 8	1.2/F	no	AS	2.5	Hazelnut, Pistachio, Walnut

Patients no.	Age (y)/sex	Macadamia allergy	Symptoms to macadamia (reported at 6y)	Macadamia SPT wheal at 6 y (Ø mm)	Allergy to other foods at 4-6 y
MT 9	4.1/M	no	AS	1.0	No other food allergies
MT 10	4.1/M	no	AS	1.5	Peanut, Hazelnut, Pistachio
MT 11	6.1/F	no	AS	4	No other food allergies
MT 12	4.1/M	no	AS	3	No other food allergies
Patient tolerant to macadamia with SPT ≤ 2.0 mm (MT 12-27)					
MT 13	4.1/M	no	AS	0.0	Cashew
MT 14	4.1/M	no	AS	0.0	Peanut
MT 15	4.1/F	no	AS	0.0	Peanut, Cashew, Almond
MT 16	4.1/F	no	AS	0.0	No other food allergies
MT 17	6.1/M	no	AS	0.0	Cashew, Walnut
MT 18	4.2/F	no	AS	0.0	Peanut, Pistachio
MT 19	4.2/M	no	AS	0.0	Peanut, Hazelnut, Sesame
MT 20	4.1/F	no	AS	0.0	Hazelnut
MT 21	4.1/M	no	AS	0.0	No other food allergies
MT 22	4.1/M	no	AS	0.0	Cashew
MT 23	4.2/M	no	AS	0.0	Peanut, Cashew, Pistachio
MT 24	6.2/F	no	AS	0.0	Cashew
MT 25	4.1/M	no	AS	0.0	Peanut, Almond, Pistachio
MT 26	4.1/M	no	AS	0.0	Peanut, Cashew, Pistachio
MT 27	6.1/F	no	AS	0.0	No other food allergies

*AS= asymptomatic

Patients' sera were grouped into patients with macadamia nut allergy (n = 8) based on SPT to macadamia nuts ≥7 mm and one of the following: a) history of objective reaction >12 months ago consistent with OFC criteria or b) parent-reported avoiding food due to allergy. To compare allergen recognition profiles between macadamia nut allergic and tree nut allergic but macadamia tolerant patients, additional sera from tree

nut allergic patients without macadamia nut allergy were used in this study and grouped into: (1) macadamia nut tolerant-sensitised individuals (n = 12) who had a macadamia nut SPT 3–7 mm and parent-reported ingestion history (eaten >1 time since age 4); (2) macadamia nut tolerant individuals without evidence of sensitisation to macadamia nut (n = 15), as defined by SPT ≤ 2 mm [Fig 5.1].

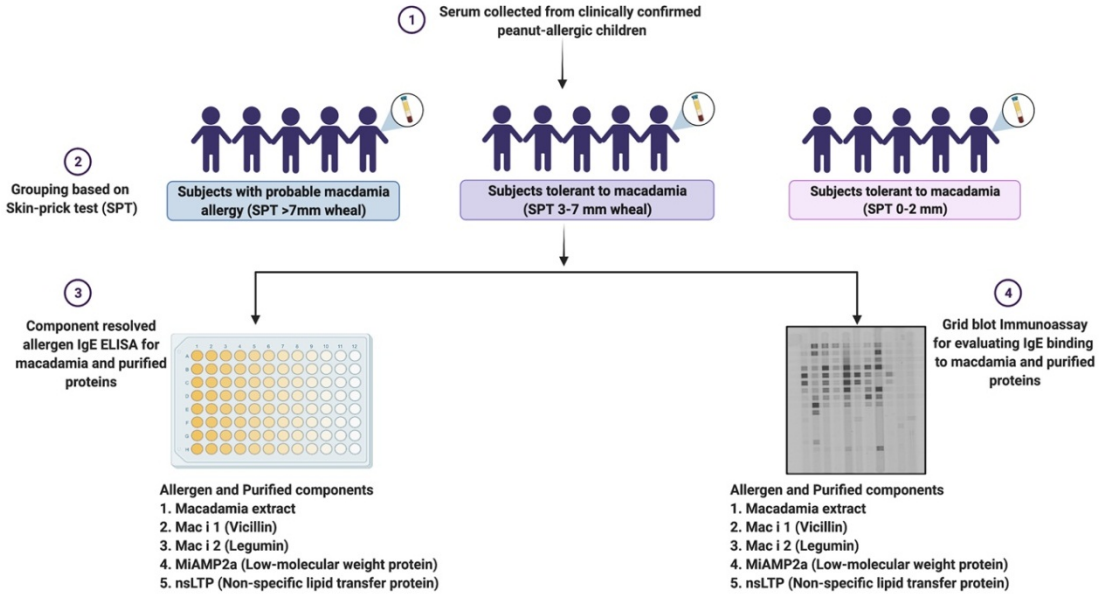

Fig 5.1: Flow diagram outlining the objectives of the study. The serum was collected from the clinically confirmed peanut-allergic individuals as well as tolerant subjects. Serum samples were later screened based on skin-prick test (SPT) results and macadamia allergy. Subsequently, allergen-specific IgE ELISA and grid-immunoassay were performed to resolve the IgE reactivity to macadamia extract and its purified proteins.

Approval for the HealthNuts study was obtained from the Victorian State Government Office for Children (reference number CDF/07/492), the Victorian State Government Department of Human Services (reference number 10/07), and the Royal Children's Hospital Human Research Ethics Committee (reference number 27047). Informed consent was obtained from parents or guardians of all participants.

5.2.2 Macadamia extract and purified proteins

5.2.2.1 Preparation of macadamia protein extract

Shelled macadamia nuts (species *M. integrifolia*) were ground and defatted with n-hexane at room temperature using a nut to solvent ratio of 1:6 (w/v). After drying,

proteins were extracted by stirring with 5 volumes of PBS, containing 3% polyvinyl polypyrrolydone and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). The slurry was centrifuged (40 000×g, at 4°C for 1 h), and the supernatant was filtered prior to chromatography.

5.2.2.2 Purification of macadamia antimicrobial peptides

The macadamia protein extract was cooled to 4°C, and ice-cold methanol was added to a final concentration of 60% (v/v) to precipitate globulins. After stirring at 4°C for 30 min, the extract was centrifuged (3000 x g, at 4°C for 45 min). The supernatant was lyophilised, and dried proteins resuspended in ddH2O before dialysing against 20 mM Tris/HCl, pH 8.0. Then, the dialysate was loaded onto a self-packed Q Sepharose chromatography column and equilibrated in 20 mM Tris/HCl, pH 8.0. Column-bound proteins were eluted by a linear gradient of NaCl from 0 to 0.5 M. Protein concentration was determined by the BCA test (Pierce, Cheshire, UK).

5.2.2.3 Purification of Mac i 1 (7S globulin)

Macadamia protein extract was prepared as described above (section 5.2.2.1) with the difference that 20 mM Tris/HCl, pH 7.5, containing high salt concentration (1 M NaCl) was used to increase solubility of globulins. The extract was loaded onto a HiPrep Sephacryl S-200 HR size exclusion chromatography (SEC) column (GE Healthcare, Uppsala, Sweden), equilibrated with the buffer mentioned above. Proteins were eluted at a 1.3 ml/min flow rate, and 5 ml fractions were collected. The fractions containing Mac i 1 were pooled, dialysed against 20 mM Tris/HCl, pH 8.0, and loaded onto a Mono Q column (GE Healthcare, Uppsala, Sweden) equilibrated in the same buffer. Proteins were eluted by a linear NaCl gradient (0-0.4 M). Mac i 1 containing fractions were pooled and subjected to a HiTrap Con A Sepharose-4B column (GE Healthcare, Uppsala, Sweden) equilibrated in 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl. Mac i 1 was eluted in one fraction by adding 0.5 M alpha methyl mannopyranoside (Sigma Aldrich, St. Louis, MS, USA).

5.2.2.4 Purification of Mac i 2 (11S globulin)

For the purification of Mac i 2, protein extraction and SEC were carried out as described in sections 5.2.2.2 and 5.2.2.3. After SEC, fractions containing Mac i 2 were pooled and applied to a Con A Sepharose-4B column. Since Mac i 2 is not glycosylated and does not bind to Con A Sepharose-4B, any residual Mac i 1 was removed by this step.

5.2.2.5 Expression and purification of recombinant macadamia nsLTP

The DNA sequence of mature macadamia nsLTP (ENA accession no: LR861101) starting at nucleotide position number 79 (5'GCCATC…) was used for recombinant protein expression. Codon optimisation, gene synthesis and subcloning of the sequence to the vector pPICZαA were performed by Thermofisher Scientific GeneArt GmbH (Regensburg, Germany). Recombinant nsLTP was expressed in the Pichia pastoris strain GS115, as described previously (Dubiela et al., 2017). The expressed protein was purified from the culture supernatant. Briefly, 800 ml of culture supernatant were lyophilised, redissolved in 20 mM sodium acetate buffer, pH 6.0 and dialysed against the same buffer. The sample was loaded onto a SP Sepharose column, equilibrated with 20 mM sodium acetate buffer, pH 6.0. Column-bound proteins were eluted by a linear gradient of NaCl from 0-0.2 M.

5.2.3 IgE ELISA

Wells of 96-well plates (Maxisorp; Nalgene Nunc International, Roskilde, Denmark) were coated with 0.2 µg of pure protein or 1 µg of protein extract, diluted in coating buffer (50 mM Na-carbonate, pH 9.6). The plates were blocked for 2 h at room temperature with TBST containing 3% (w/v) BSA. Subsequently, patients' sera (diluted 1:10 in TBST containing 1% (w/v) BSA and 100 µg/mL horseradish peroxidase to block antibodies specific for cross-reactive carbohydrate determinants) were applied in duplicates overnight at 4°C. Detection of bound IgE was performed with alkaline phosphatase (AP)-conjugated mouse anti-human IgE antibody (BD Pharmingen, San Jose, Ca, USA) followed by incubation with Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St Louis, Mo, USA). Absorbance at 450 nm was measured.

Sera of four non-atopic donors served as negative controls. Sera were regarded as positive if their OD exceeded the mean OD value of the four healthy controls plus three times their standard deviation.

5.2.4 Grid-blot immunoassay

A grid-blot immunoassay technique (modified from Reese et al. (2001) was performed to examine the presence of allergen-specific IgE in patient serum to determine sensitisation patterns to macadamia extract and purified proteins[28]. The assay was performed using a surf blot apparatus (Idea Scientific, MN, USA) assembled according to the manufacturer's instructions. Nitrocellulose membrane (Bio-Rad, USA) was immobilised in the apparatus as instructed, and either 10 µg of protein extract or 2 µg of purified allergen in 200 µl coating buffer (50 mM Na-carbonate, pH 9.6) was injected into each channel. The membrane was incubated for 1 h at room temperature with end-to-end rocking. Later, the membrane was blocked using 1x Casein (Casein Blocking Buffer 10x, Sigma, VIC, Australia) in PBS with 0.05% Tween-20 (PBS-T) (Bio-Rad, USA). After the blocking step, the membrane was rotated to 90° as instructed to align the grids for serum incubation. Subsequently, the membrane was incubated overnight with patient serum diluted as 1:20 in antibody buffer (0.5x Casein + 100 µg/ml HRP in PBS-T) solution. The following day, the serum solution was removed, and the membrane washed carefully with PBS-T solution to minimise bleaching and unspecific binding. Later, IgE binding was detected using polyclonal rabbit anti-human IgE antibody (diluted 1:20,000 in 0.5x Casein) (Dako, Glostrup Denmark) prepared in PBS-T. After 1 h incubation secondary goat anti-rabbit IgG antibody (1:20,000 diluted in 0.5x Casein) (Dylight™ 800, Thermo, IL, USA) was used. Antibody binding on the membered was visualised using the Odyssey CLx Imager (Li-cor, NE, USA). The band intensity was computed using the Image Studio™ software. The final band intensity values were normalised against the local membrane background and exported into Microsoft Excel. The imported data was analysed using GraphPad Prism (version 8.2) to plot a heat map with Log10 of the IgE binding intensity values against each extract/allergen component.

5.3 Results

5.3.1 Mac i 1, 2 and MiAMP2a represent relevant IgE-binding components in macadamia nut

The IgE-reactivity of macadamia extract and the purified proteins was assessed by IgE ELISA using sera from 8 macadamia allergic subjects, 8 macadamia nut tolerant but sensitised group and 14 non-sensitised group [Fig 5.2].

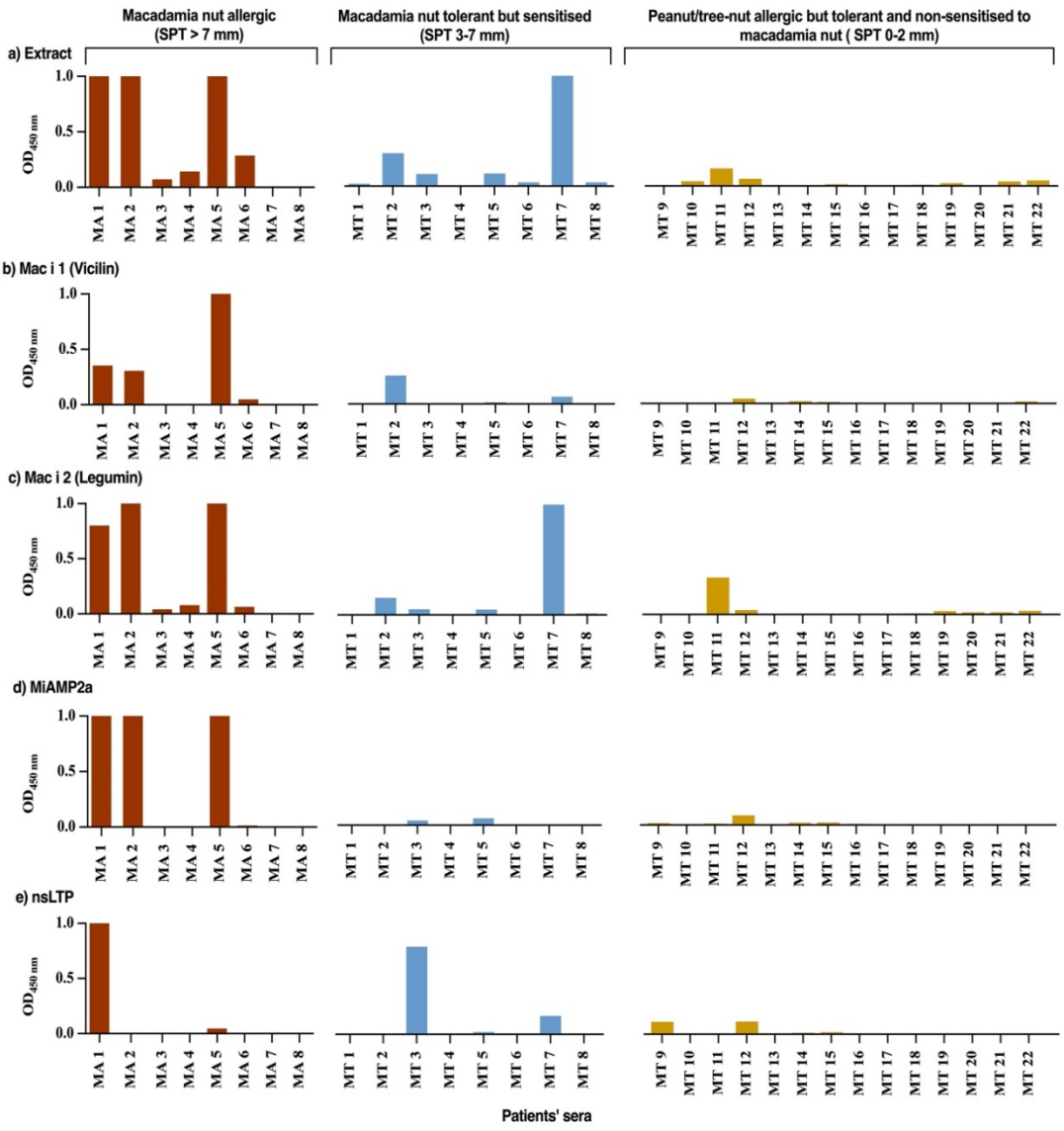

Fig 5.2: ELISA analysis of IgE binding to macadamia extract (a), Mac i 1 (b), Mac i 2 (c), MiAMP2a (d), and nsLTP (e). IgE binding to purified components was analysed using sera from macadamia allergic (red bars), tolerant-sensitised (blue bars) and SPT-negative (yellow bars) subjects. Sera were deemed positive if they exceeded the mean OD value of four healthy controls plus three times their standard deviation.

In macadamia allergy groups, 75% (6/8) had IgE to macadamia extract. Specific IgE to purified vicilin (Mac i 1) was detected in 50% (4/8) of tested sera. Macadamia legumin (Mac i 2) and MiAMP2a were recognised by IgE from 75% (5/8) and 38% (3/8) of patients' sera, respectively. Sensitisation to recombinant nsLTP was only observed in 25% (2/8) of macadamia-allergic patients. In the macadamia control group of SPT-positive but clinically tolerant patients (n=8), the IgE-binding frequency to extract was 75% (6/8), to Mac i 1 25% (2/8), to Mac i 2 50% (4/8) and to MiAMP2a 25% (2/8). Sensitisation to nsLTP was seen in 25% (2/8) of macadamia tolerant patients. Notably, 36% (5/14) and 14% (2/14) of subjects without macadamia allergy and a negative SPT result had sIgE to extract and Mac i 2, respectively. However, only 7% (1/14) of these subjects were sensitised to Mac i 1 and MiAMP2a, respectively.

5.3.2 IgE reactivity to macadamia extract and purified proteins

In this cohort, there were only 8 subjects with self-reported and SPT confirmed allergic reactions to macadamia nut and 27 subjects either tolerant but sensitised or non-sensitised to macadamia with SPT values ranging from 3-5 mm (MT 1-12) and 0-2 mm (MT 13-22). Grid-blot immunoassay was conducted to evaluate the IgE reactivity of participants against macadamia extract and its purified proteins (Mac i 1, Mac i 2, MiAMP2a, and nsLTP) [Fig 5.3]. Cross-reactive IgE-binding reactivity to macadamia extract and purified proteins were presented as a heatmap with the colour scale ranging from white (no binding) to blue (high binding) [Fig 5.4]. The IgE binding to macadamia extract was found to be high in 25% (2/8) of the macadamia allergy group (SPT >7), with the majority (75%, 6/8) showing moderate IgE binding. Notably, 33% (4/12) of the macadamia tolerant group (SPT 3-7) showed higher IgE binding, and 77% showed moderate to low binding to macadamia extract. In the macadamia non-sensitised group 26% (4/15) showed moderate IgE binding to whole extract with 73% (11/15) showing low IgE bindings towards macadamia extract.

For Mac i 2 (legumin), 12.5% (1/8) of subjects had low IgE binding as opposed to the macadamia tolerant group with 16.6% (2/12) showing high IgE binding and 25% (3/12) showing low IgE binding. Notably, in the non-sensitised group, 26.6% (4/15) of subjects had moderate, and 66.6% (10/15) had low IgE binding to Mac i 2.

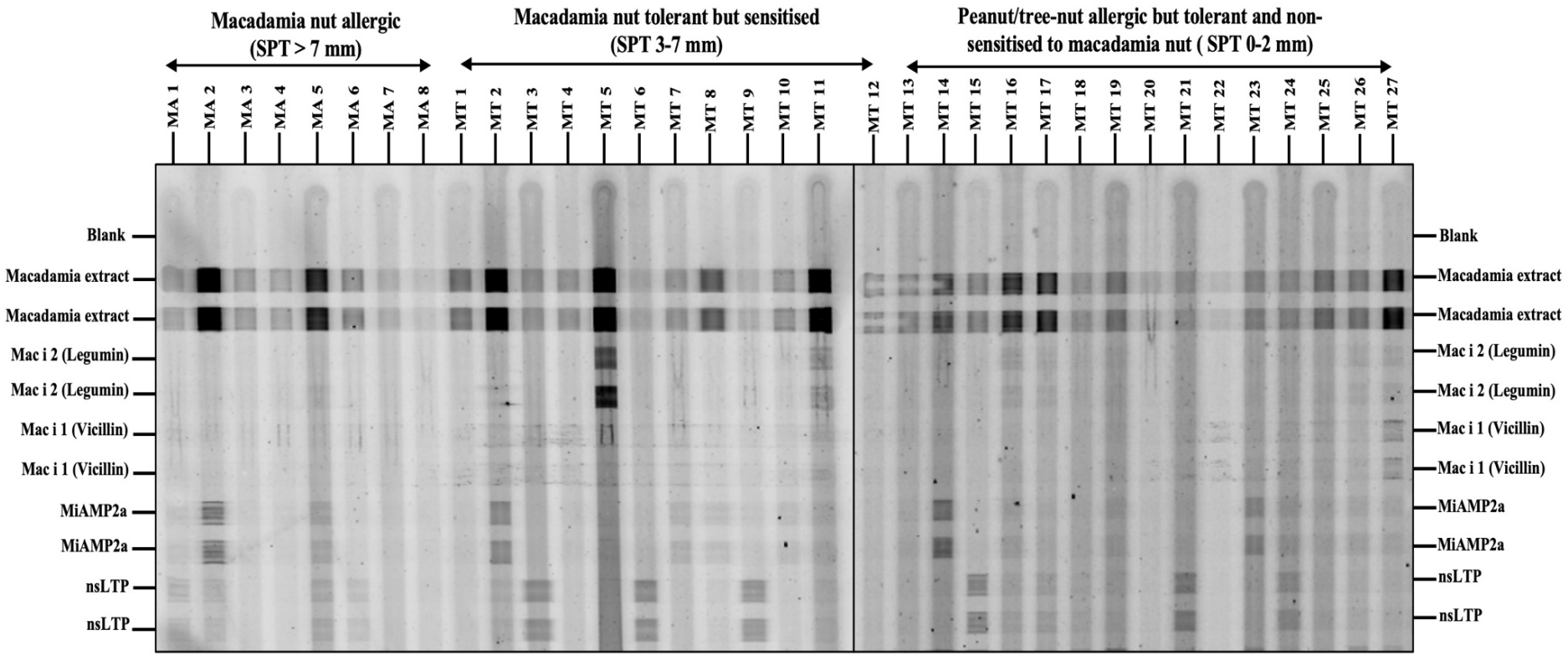

Fig 5.3: Grid-blot immunoassay displaying the specific IgE reactivity to macadamia extract and its purified allergens (Mac i 1, Mac i 2, MiAMP2a, and nsLTP), in duplicate, using macadamia allergic patient serum (MA, 1-8), tolerant groups (MT 1-12, MT 13-27). The groups were distributed based on skin-prick test values. The membrane was processed using ImageStudio software. MA: macadamia allergy, MT: macadamia tolerant, Mac i 1: vicilin, Mac i 2: legumin, MiAMP2a: lower molecular weight protein, nsLTP: mon-specific lipid-transfer proteins.

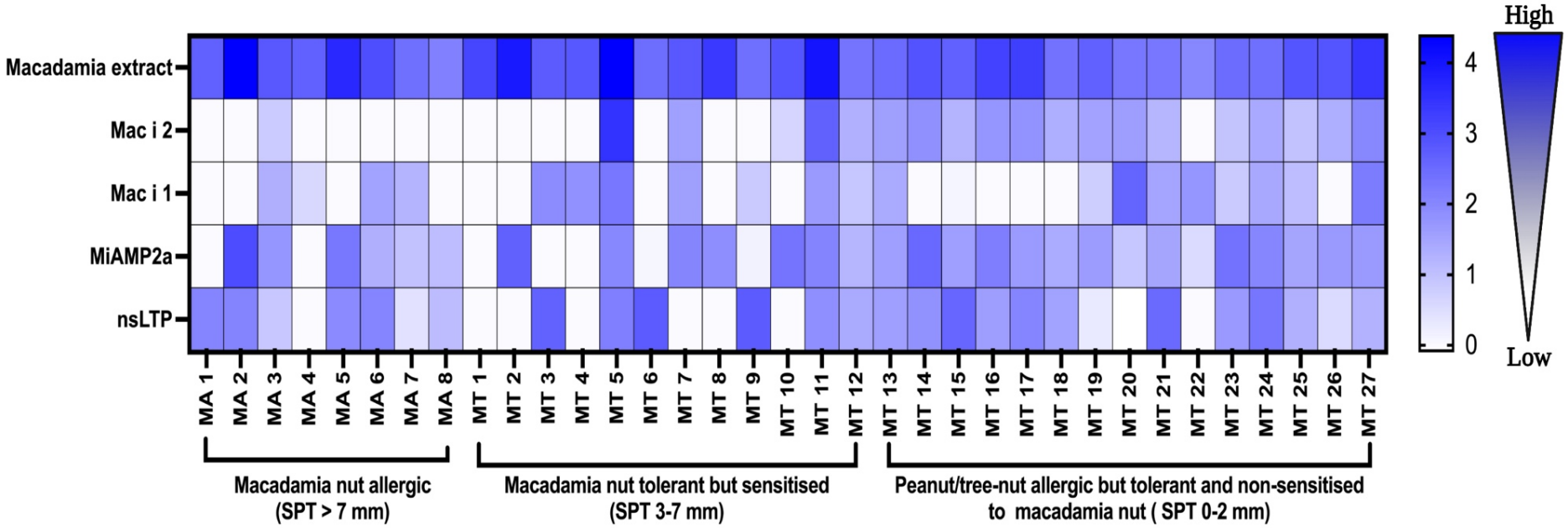

Fig 5.4: A heatmap displaying the specific IgE reactivity to macadamia extract and its purified allergens (Mac i 1, Mac i 2, MiAMP2a, and nsLTP) in macadamia allergic subjects (MA, 1-8) and control groups (MT 2-12, MT 13-27). The groups were distributed based on skin-prick test values. The average binding intensity is represented as the log10 of the IgE binding signal measured using ImageStudio software. Intensity is represented in levels ranging from 0-4 (lower binding intensity to higher binding intensity). The binding intensity is expressed in colour from white (no binding) to dark blue (strong binding). MA: macadamia allergy, MT: macadamia tolerant, Mac i 1: vicilin, Mac i 2: legumin, MiAMP2a: lower molecular weight protein, nsLTP: non-specific lipid-transfer proteins.

Furthermore, 50% (4/8) subjects of the macadamia allergic group showed low IgE binding to Mac i 1 (vicilin). In addition, low to moderate IgE binding was observed in 58.3% (7/12) subjects in the macadamia tolerant group, whereas the non-sensitised group had 6% (1/15) high and 46.6% (7/15) low to moderate IgE binding to Mac i 1.

IgE binding to MiAMP2a protein in the macadamia allergy group was 12.5% (1/8) with high IgE binding and 5/8 (62.5%) with low to the moderate binding. Subsequently, in the macadamia tolerant group, 25% (3/12) of subjects reacted to MiAMP2a with moderate IgE binding, and 33.3% (4/120) showed a low IgE binding pattern. In the non-sensitised group, 26.6% (4/15) subjects had moderate binding, whereas 73.3% (11/15) had low binding. In the macadamia allergic group, 50% (4/8) subjects showed moderate IgE binding to nsLTP protein, with 25% (2/8) showing low binding. Interestingly, in the macadamia tolerant group, 25% (3/12) subjects showed high IgE binding, and 25% (3/12) showed low IgE binding to nsLTP protein. Notably, in the non-sensitised group also had 20% (3/15) of subjects had moderate IgE binding and 60% (9/15) showing low IgE binding to nsLTP protein. Most subjects in all three groups showed a distinct IgE reactivity pattern to macadamia extract and purified proteins.

5.4 Discussion

In the past decade, a significant interest in purified and well-characterised allergens has increased exponentially due to the rise in research efforts on immunotherapy-based approaches[29]. Diagnostic approaches in molecular allergy based on the allergen-specific measurement of IgE levels against specific culprit food proteins provide crucial information in diagnosing food allergy[30]. Out of big-8 food allergen sources, peanut and tree nuts share a substantial portion of food allergy incidences around the globe[2]. A significant amount of information on peanut allergens is available; however, up to date knowledge of the allergens involved in macadamia allergy is lacking[5]. In this study, we identified major and minor IgE-binding proteins in macadamia nut, which may be used as marker allergens to facilitate patient-tailored food allergy management and evaluate the risk of cross-reactivity with peanut or other tree nuts.

The prepared macadamia nut extract generated in this study and individual, purified proteins were analysed for IgE reactivity using ELISA. In our tested cohort, 75% of patients with confirmed macadamia allergy had sIgE to the whole protein extract. The two patients with a negative test result are likely sensitised to additional IgE-binding macadamia nut components (e.g., Bet v 1 homologue, profilin and oleosins), which could be missing in our extract. The IgE sensitisation prevalence to vicilin and legumin were 50% and 75%, respectively. Previously, Ehlers *et al.* reported a positive correlation between the severity of the allergic reaction and allergen-specific IgE level when they expressed the full-length vicilin precursor, resulting in 30% IgE-binding frequency among macadamia nut allergic patients[16]. In our study, instead of using the recombinant full-length precursor, we tested our patient cohort with purified post-translationally processed mature vicilin from the natural source. The higher sensitisation frequency in our cohort may result from relevant conformational IgE epitopes formed during the processing and folding of the mature protein. Three of the four patients with a positive IgE reaction to mature vicilin were co-sensitised to MiAMP2a, suggesting an important IgE-binding role of this specific processing product.

In contrast, the IgE-sensitization rate measured using ELISA to recombinant nsLTP was lower among macadamia nut allergic patients. This observation might be explained by the fact that patients' sera from an Australian cohort were used in this study. In general, nsLTP sensitisation is mainly associated with the Mediterranean area[31]. In order to evaluate the relevance of sensitisation to macadamia nsLTP, further studies are required to be conducted in the Mediterranean region.

In contrast to the above, grid-blot immunoassay performed to measure the cross IgE reactivity to macadamia extract and its purified proteins presented varied outcomes. In the macadamia allergic patient cohort, only 25% of subjects had high IgE binding, contrasting with 75% of patients showing low to moderate IgE binding. Similarly, in macadamia sensitised and non-sensitised groups, moderate to low binding was noted in immunoassay. Furthermore, contrasting results in Mac i 1 (vicilin) and Mac i 2(legumin) groups were also observed with macadamia allergic patients showing low binding (50% and 12.5%, respectively) as opposed to moderate to low binding observed in sensitised and non-sensitised groups. MiAMP2a and nsLTP IgE binding patterns

revealed similar results. Therefore, these results reveal a striking difference between the techniques performed to measure IgE binding. However, the different binding pattern on ELISA and grid-blot immunoassay could be in part explained due to the two different binding surfaces, i.e., 96-well plate and nitrocellulose membrane. The surface chemistry, different types of antibodies used in the assay and matrix affects with different proteins could result in differences in available epitopes or moieties for interacting with serum IgE antibodies.

Apart from the differences in IgE binding between ELISA and grid-blot immunoassay, the outcomes from the study confirm the cross-reactive nature of macadamia nut extract and proteins with patients allergic to peanut and sensitised to other tree nuts. Among the low molecular weight proteins present in tree nuts and peanut, members of the prolamin superfamily, including 2S albumins and nsLTPs, play an important role in allergic disease. In particular the 2S albumins from hazelnut (Cor a 14), walnut (Jug r 1), cashew (Ana o 3), and from peanut (Ara h 2) have been shown to correlate with the severity of allergic reactions[24,32–34]. Sensitisation to nsLTPs is associated with mild to severe symptoms and mostly restricted to distinct geographical regions[35].

Our data suggest that macadamia extract and its purified proteins are IgE reactive with macadamia allergic, sensitised, and non-sensitised patients to a varying degree from high to low binding patterns as reflected in a cohort of clinically confirmed peanut or tree nut allergies. Therefore, the identified macadamia proteins especially may represent a marker of macadamia allergy and cross-reactivity with peanut, with possible application in molecular diagnosis. However, given the monocentric study design and the relatively small number of serum samples available of macadamia allergic individuals, our findings need to be confirmed in future investigations, including other geographical populations and larger cohorts. Future studies will be required to assess the extent of cross-reactivity with other tree nut species and whether different sensitisation patterns correlate with the severity of clinical manifestations of macadamia allergy. The well-characterised macadamia allergens described in this study may help with diagnosis of food allergy and patient-specific dietary recommendations.

5.5 Summary

Chapter-5 characterises macadamia extract and purified allergens isolated from macadamia nut and further evaluated their IgE reactivity in a cohort of Australian individuals with clinically confirmed peanut or tree nut allergy. Chapter-2 and 3 focused on developing and optimising a mouse model for peanut allergy to investigate potential natural therapeutic options in the form of extract and purified polysaccharides isolated from green macroalgae *Ulva ohnoi*. However, peanut as a nut belongs to the family of legumes and resembles closely in terms of its protein composition to that of tree nut. Therefore, peanut-allergic individuals could develop a cross-reactive allergic disposition towards tree nuts. Chapter-5 explores the investigation on macadamia nut, a native nut utilised in food and bakery products, as a potential food allergen source that likely could instigate a cross-reactive allergic reaction in peanut-allergic individuals.

The results in Chapter-5 report significant cross-reactive IgE binding to macadamia extract and its purified proteins as measured using ELISA and grid-blot immunoassay. Despite the differences in the binding patterns between the two techniques, IgE antibody was shown in macadamia allergic, macadamia sensitised, and macadamia non-sensitised patient serum samples. Our findings highlight that the proteins isolated from macadamia nut could assist in the improved diagnosis of nut allergy. In conclusion, macadamia extract and its purified proteins can be utilised in future investigations to identify individuals with food allergies to specific proteins in macadamia nuts.

5.6 References

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Chapter-6

General discussion & future directions

6.1 Overall Summary

This thesis aimed to investigate the effect of algal polysaccharides as a novel therapeutic option for food allergy. Based on the previous literature on the natural compounds possessing anti-allergic properties, I hypothesised that algal extract or purified polysaccharide isolated from the marine algae *Ulva ohnoi* (*U. ohnoi*) could serve as an alternative natural therapeutic for the treatment of food allergies. Existing preclinical mouse models were identified based on peanut allergy in the existing literature for studying the effect of algal extract and purified polysaccharides on the pathophysiology and mechanism of an established food allergic reaction. A comprehensive literature review was performed to identify the existing evidence on the effects of various algal compounds and isolated polysaccharides on different disease models, including allergy models (Chapter-1). Based on the previous literature, I established an intragastric route-based allergy model to induce peanut allergy in mice; however, I failed to establish a sustained allergic response in female BALB/c mice (Chapter-2). By tracing the steps, potential variables were identified that could have caused the failure in establishing an allergy model. I found that the length of the immunisation protocol, route of sensitisation, type of housing facility and changes in the microbiome were underlying variables that caused a shift in Th1/Th2 responses during the induction of peanut allergy (Chapter 3). After establishing a working preclinical model, I decided to check the biological efficacy of feeding purified ulvan polysaccharides to mice for a prolonged period and its effects on the gut microbial community structure. I used a well-established carotenoid (astaxanthin) as a reference molecule to compare the results. It was shown that feeding ulvan polysaccharide isolated from *U. ohnoi* positively modulates the gut microbial community structure in mice comparable to astaxanthin's that may assist in developing immune tolerance (Chapter 4).

Furthermore, peanut is one of the Big-8 allergens responsible for causing increasing incidences of allergic reactions around the globe[1]. Existing literature reports that the protein composition of peanut shares similarities with various other tree nut allergens,

including Macadamia nut[2]. Macadamia nut consumption is highest in Australia compared to the rest of the world, and it shares a significant amount of protein similarity with peanut[3]. Due to the protein similarity, the chances of presenting macadamia nut proteins as allergens to the peanut-allergic patient increases exponentially[3]. Therefore, I designed a study to evaluate the IgE reactivity in a cohort of Australian individuals with clinically confirmed peanut or tree nut allergy against macadamia nut extract and purified allergens. We found a significant cross-reactive IgE binding to macadamia extract and its purified proteins as measured using ELISA and grid-blot immunoassay (Chapter 5). The outcomes from this thesis present an argument for the use of algal polysaccharide as a natural therapeutic option for balancing Th1/Th2 response and the gut microbiota to mitigate allergic response in a mouse model. Additionally, this thesis demonstrated that preclinical models used in the allergy studies require optimisation based on various macro- and micro-environmental variables, as pointed out in the study conducted in Chapter-3.

Furthermore, the purified ulvan extract from *Ulva ohnoi* used in this thesis will have a very different regimen and associated guidelines if translated for human consumption. The feeding of the purified ulvan as a treatment would require daily consumption either as a preventative source or treatment in an allergy-based scenario. Additionally, the patients would also require continuing avoidance of the allergen source. Mouse to human translation require upscaling of the dose hence there will be an increased consumption threshold while on treatment and will need to be maintained following cessation of treatment due to the possibility of relapse. This thesis acknowledges there are limitations and difficulties of translating the outcomes presented in this thesis to human consumption-based option; however, the use of natural algal polysaccharides presents an enticing avenue to use them as potential alternative therapeutic or nutraceutical approaches.

In this final chapter, I will be discussing the implication and limitations to the outcomes of this thesis in the context of the current research scenario. Additionally, I will discuss the future directions and generalisability of the research outcomes in this thesis.

6.2 Algal extract and polysaccharides to treat peanut allergy

The prevalence of food allergy is increasing and poses a more significant threat to those afflicted [4]. Amongst the Big-8 allergen sources responsible for food allergy, peanut is the most commonly reported allergen source worldwide[1]. Despite the increase in clinical reports and prevalence of peanut allergy, there remains a lack of a general and holistic therapeutic approach to treat peanut allergy[5]. The current research is centred around specific, allergen-based immunotherapies that cannot treat or suppress allergic responses in people affected with multiple food allergies[5]. Presently there is only one FDA approved therapeutic option available for the treatment of peanut allergy; however, due to the specific nature of immunotherapy, the success rate of it working for every patient is relatively low[6,7]. Additionally, the current management option available for people with peanut allergy is completely avoiding the offending source and carrying emergency medications such as EpiPen (auto-adrenaline injector) in case of accidental exposure[8]. Consequently, the individuals and their family members are affected physically because of existing food allergies, for that matter and suffer at an emotional level due to social isolation, fear and worries of accidental exposure in a social setting[9,10].

As discussed in Chapter-2, marine algae are a potent source of carbohydrates, proteins and various other minerals and vitamins. The overall composition constitutes a large portion of carbohydrates made up of polymeric units of sugar known as polysaccharides[11]. Marine algal polysaccharides have been reported to possess anti-allergic properties such as anti-asthmatic, anti-rhinitis and suppression of airway hypersensitivity[11–14]. Out of different algae, namely, brown (*Phaeophyceae*), red (*Rhodophyta*) and green (*Chlorophyta*) algae, green algae are unexplored and underutilised for investigating their role in suppressing various disease states. In Chapter-2, I utilise a crude extract and a purified ulvan from green macroalgae *U. ohnoi* to explore a potential natural therapeutic option for suppressing peanut allergy in a mouse model. A pilot trial was conducted for evaluating the toxicological effects of crude extract and purified polysaccharide before developing the allergy model. The

feeding of crude extract or purified polysaccharide had no significant effects on biochemical enzymes. The underlying hypothesis behind the study was to establish a suppressive response in a peanut allergy model due to the feeding of crude extract and purified ulvan. The purified ulvan did represent a trend of balancing the Th1/Th2 response to suppress an allergic response. However, admittedly a sustained allergic response was not developed in mice which inhibited the estimation of biological efficacy to a considerable extent. Although the mouse model did hinder the study, a trend in the data was observed where that data shows the potential of ulvan extract and, more specifically, purified ulvan to be effective in a well-established model of peanut allergy.

The idea of using marine algal extract or purified polysaccharides to suppress an allergic response provides an avenue to explore a more holistic approach instead of a targeted one. The efficacy and potential of algal extract and ulvan can also be further explored in the context of other allergen sources such as tree nuts, shellfish, fish, and egg allergy. The cost-effectiveness and widespread use of marine algal extracts is an open opportunity to develop broad-spectrum therapeutic options.

6.3 Mouse models: a mine full of variables

Laboratory mice are a fundamental backbone of basic biomedical research and have been a crucial part of some instrumental discoveries in the field of basic immunology[15]. However, there are major underlying limitations, including reports of contradictory findings based on gut microbiota due to research facility differences, difficulty in predicting immune responses that closely resemble human disease states, different induction methodologies, and varying strains of mice[15–17]. Recent studies suggest that the specific pathogen-free facility (SPF) is far too different from the conventional housing facilities of mice due to controlled natural environmental conditions in the former, which fails to mirror the housing and physiology of mammals such as humans[16]. We thrive and evolve based on the surrounding microbial world and behave

differently physiologically and immunologically in a controlled and sanitised environment[18].

As discussed in Chapter-3, based on the outcomes from Chapter-2, a mouse model of peanut allergy was developed and compared in two different housing facilities, SPF, and conventional facility. The outcomes from the study suggest that conventional facility housed mice mirrored the clinical (drop in body temperature) and immunological responses (total IgE, sIgG, sIgG1 and sIgG2a) of a typical allergic reaction in mice as opposed to SPF facility. Therefore, a mouse model of allergy developed in a conventional housing facility could be a more suitable option for future research. Additionally, two separate routes of sensitisation, i.e., intraperitoneal (IP) and oral gavage were used to induce the peanut allergy. The results showed a significant difference between the IP and oral sensitisation, indicating that the oral route of induction used in Chapter-2 could be the likely cause of an unsustained allergic response.

Furthermore, mice housed in two different facilities had distinct microbial community signatures, meaning the conventional facility had a more enriched microbial community structure than the SPF facility. Previous studies have reported changes in the microbiome of mice housed in two different facilities[16]. Recent reports also indicate that germ-free mice could have impaired mast cells that contribute to the failure to develop an allergic response in a food allergy model[19].

Mouse models are used in basic and translational immunology research and are an integral part of allergy research[20]. However, based on the results presented in this thesis, the optimisation of mouse models of food allergy is required. In practicality, there is no way to standardise a mouse model with so many variables across research institutions. However, a recent report by Marion *et al.* suggests that gnotobiotic mice could capture the SPF or specific (and opportunistic)-pathogen-free (SOPF) phenotype when a 15-member bacterial load having a bacterial population from *Bacteroides*, *Clostridia,* and *Lachnospiraceae,* among others, is introduced while developing a mouse model[21]. Another study by Susan *et al.* suggests a different methodology of developing a cross of

two different strains of mice procured from two different facilities could have standardised microbiota in the littermates[22]. Perhaps, developing the mouse model while noting the physiology and microbiome-influencing factors in a multi-site study could mitigate variability and make it easier to track the effects of different variables that led to unexpected outcomes.

6.4 Modulating the microbiome using green algae

The gut microbiome is an intricate ecosystem consisting of thousands of microbial species and individual species variations[23]. The role of gut microbiota in the modulation of metabolic functions and host immune system is well documented[24,25]. Out of thousands of bacterial species, the bacteria belonging to the phylum of *Bacteroidetes* and *Firmicutes* make up 90% of the intestinal composition out of the entire phylogenetic type found in the intestine[25]. A balanced microbiome is a paradox; however, it is believed that there is an equilibrium state between the intestinal microbes and the host, which is dynamic in nature[25]. The dysbiosis of the said balance leads to the disease state and affects the gut microbiome and metabolic functions[25]. Therefore, a natural source-based approach to maintaining the balanced state could prove beneficial in establishing the proper physiological functions of the host, regulating immune response against intruding pathogens[8].

As discussed in Chapter-4, ulvan polysaccharides isolated from green macroalgae *U. Ohnoi* and carotenoid isolated from freshwater microalgae *Haematococcus Pluvialis* (*H. Pluvialis)* were found to be increasing the abundance of the *Bacteroides and Firmicutes* along with the population of *Clostridia* known for their probiotic functionality. In an allergy state, the gut microbiome is affected due to the mounting of an inflammatory response leading to gut microbial dysbiosis[8]. The microbial dysbiosis increases the number of antigen-presenting cell populations in the gut, causing a cascade of inflammatory responses and reducing the mucus-producing goblet cell population[15]. However, feeding the ulvan polysaccharide shows an increase in the gut microbial population of *Bacteroides* and *Firmicutes,* i.e., assisting mucus-producing bacterial population[26]. The outcomes of this study could imply that the gut microbial equilibrium could be maintained by feeding algal polysaccharides. A higher abundance

of *Clostridia* upon feeding algal polysaccharides also indicates the potential of probiotic functionality that could be further explored in future studies[26]. Tending the microbiome using natural means such as green algae or their purified compounds could be a potential therapeutic option with a wide range of applications.

6.5 More nuts to crack to study the allergy paradigm

Peanut allergy contributes to food-induced anaphylactic fatalities worldwide; however, tree nut allergens pose a significant threat due to the protein similarity with peanut allergens[2]. Due to the protein similarity, peanut allergens cross-react with other legumes and tree nut allergens[27]. Peanut allergic individuals most commonly exhibit sensitisation to the allergens from these other sources; however, cross-reactivity to these allergens is not always clinically relevant as exhibited by skin-prick tests[28]. As shown previously, half of the peanut-allergic patient cohort presented with the positive skin prick test to legumes; however, only 5% amongst them were found to be clinically allergic[29]. The major concern is this 5% population that could potentially cross-react to the legumes and other tree nut allergens. Among these tree nut allergens, the macadamia nut is widely consumed in Australia and exported to other parts of the world. However, studies of the macadamia nut and its purified proteins are not extensively reported for their cross-reactivity.

As discussed in Chapter-5, an Australian cohort of peanut-allergic and tree nut sensitised patients was used to study the IgE reactivity using *in vitro* approaches such as ELISA and grid blot immunoassay. The outcomes presented in the chapter showed that about 97% of the cohort showed IgE binding to macadamia nut extract irrespective of their clinical history. As patients with existing peanut allergies, the risk of developing a cross-reactive allergic reaction to tree nuts such as macadamia is high and could also potentially lead to anaphylaxis. Therefore, individuals with existing nut allergies must avoid exposure or ingesting other tree nuts and legumes.

Furthermore, the study also investigated the IgE reactivity to component allergens isolated from macadamia nuts. The outcomes showed that the macadamia allergic patient cohort selectively exhibited IgE reactivity to specific allergens, whereas macadamia tolerant and sensitised groups exhibited low to moderate reactivity. In

brief, three allergen proteins [vicilin (Mac i 1), legumin (Mac i 2), macadamia antimicrobial peptides (MiAMPs)] were extracted from macadamia nut, and a recombinant non-specific lipid transfer protein (nsLTP) was used to conduct allergen-specific ELISA detection. All five sera from macadamia allergic patients had IgE specific to Mac i 2 (legumin), and four had IgE specific to Mac i 1 (vicilin)[3]. Three of the four patients with a positive IgE reaction to mature vicilin were co-sensitised to MiAMP2a, suggesting an important IgE-binding role of this specific processing product[3]. Based on our findings, the vicilin (Mac i 1) and the legumin (Mac i 2) were designated novel allergens by the WHO/IUIS Allergen Nomenclature Sub-Committee. MiAMP2a was additionally listed as Mac i 1.0101 (28–76) as an individual IgE-binding moiety derived from the vicilin precursor (www.allergen.org)[3].T The registration of specific allergens could be a step in the right direction for optimising the *in vitro* diagnostic approaches that could assist in the early detection of specific allergen sources behind food allergy incidences and thereby assist in making an informed decision while registering the clinical history of patients and while conducting future interventions.

6.6 Overall study limitations

The research conducted in this thesis was centred around the hypothesis based on preclinical investigations to study the anti-allergic properties of marine green algae extract and isolated compounds using a mouse model of food allergy. There are advantages to the preclinical animal model for studying basic immunology and the disease mechanism; however, there are also limitations to them. The limitations associated with different chapters are discussed below:

1. The study in Chapter-2 was designed using female BALB/c mice to induce peanut allergy using cholera toxin B as an adjuvant. However, the model failed to mount a sustained allergic response. The limitations such as length of sensitisation, route of immunisation and microbiome changes due to clean housing facility were later investigated in Chapter-3 to optimise the mouse model of peanut allergy.

2. Additionally, in Chapter-2, the study was designed to check the biological efficacy of algal extract and ulvan polysaccharide isolated from green algae. However, the Th2 response suppressing potential could not be estimated due to the failure to establish the sustained allergic response.
3. The study in Chapter-3 involved the optimisation of a mouse model of peanut allergy based on the outcomes observed in Chapter-2. The model was developed with two different routes of sensitisation in two different housing facilities. The results indicated a more clinically accurate allergic response manifestation in conventional housing compared to SPF facility. However, the study was limited to one single strain of mice. Previous studies have indicated that strain differences (C57BL/6J, BALB/c, C3H/HeOuJ) could have varying responses based on the route of sensitisation[16,30].
4. The study in Chapter-4 involved feeding ulvan polysaccharide and astaxanthin, a carotenoid, to mice and investigating their effect on the gut microbiome. However, the study was limited to 16s rRNA based microbiome analysis. A more in-depth analysis using a large sample size and whole-genome sequencing (WGS) can be performed to study the effects on the gut microbiome. Furthermore, the study was designed on naïve animals with a single strain of mice; therefore, no reference microbiome could be established for comparison. A different strain of mice has varying microbial signatures; therefore, an extensive study on different strains of mice can be performed to fully understand the overall effect of algal polysaccharides or carotenoids on the gut microbiome. The outcomes can later be used as reference data for preclinical models to study cause and effect mechanisms based on different diseases.
5. The study in Chapter-5 utilised patient sera from peanut allergic patients reported to be either macadamia allergic, tolerant, or non-sensitised was used to compare the IgE reactivity to macadamia nuts and purified allergens. The study was limited in the number of macadamia allergic patients available for the study compared to the tolerant or sensitised group. Furthermore, the results showed cross-IgE reactivity to macadamia extract and allergens, vicilin (Mac i 1), legumin (Mac i 2), macadamia antimicrobial peptides (MiAMPs) and nsLTP, irrespective of clinical history. Further optimisation in selecting the patient

cohort is required to characterise the culprit allergens, as this could prove beneficial in optimising the *in vitro* diagnostic approaches.

6.7 Future directions

Global demand for the treatment of allergies is ever-growing. Therefore, natural algal compounds used as an alternative therapeutic approach could be considered beyond their traditional role in regulating nutritional health. However, the fundamental question that remains to be answered is how these natural treatment regimens can be used to induce long term tolerance with a profound reduction in IgE level with acute safety testing.

Since to test the efficacy of these algal compounds as preventive measures for food allergy, an optimised model is essential to assess their safety testing by mimicking more clinically relevant symptoms. Our research suggests optimising the animal model of allergy before investigating the efficacy of any natural compound to deduce the more direct effects. One of the things that our research highlights are that food allergy, such as peanut allergy in our case, is associated with gut health. In many previous studies, along with our research, it has been recognised that various stages of food allergy development, from getting susceptible to allergens leading to the development of allergic disease, could be regulated by changes in the gut microbial community. Thus, feeding the most promising molecules of marine algal sources, i.e., polysaccharides or dietary fibres, could provide a source of potential prebiotic efficacy due to their fermentation by colonic bacteria into short-chain fatty acids (SCFA's). However, another critical aspect, i.e., to investigate the precise mechanism of host immune-microbiome crosstalk during consumption of algal extracts to develop these natural compounds based targeted therapeutics.

Besides their role as prebiotics, it is essential to study their role as a source of beneficial marine algae and natural potent treatment regimen for other diseases such as cancer, liver malformations and any other hypersensitive disorders. It is essential to explore the scale-up processing of natural polysaccharides further to implement their use in health domains with broader applications. Moreover, since they enhance the abundance of

beneficial gut bacteria, as noted in our study, they could be used as a natural source of immunity-booster through regulating the gut-immunity axis.

Our research provides an overview of the significance of the consumption of algal extract for the treatment of peanut allergy. However, their potential as algal-based prebiotics could be explored for other types of food allergy and other allergic diseases like asthma. Like red algae-derived polysaccharides, the ulvan based extract could provide an anti-allergenic effect by potentially regulating immune markers related to allergy. In recent years marine algae have been an active source of nutraceutical products or compounds with therapeutic potential. However, based on the current knowledge, there is a long way to overcome the challenges and limitations to study their impact on the host immune system, metabolism, and microbe-immune interactions to develop a more targeted natural treatment regimen.

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