

Allergenicity in food allergy
Influence of food processing and
immunomodulation by lactic acid bacteria

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Thesis

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Voor Ingrid

List of abbreviations and symbols

AA	Amino acid	IMDM	Iscove's Modified Dulbecco's Medium
Ab	Antibody	IT	Immunotherapy
ACB	Allergen challenge buffer	Kd	Dissociation constant
Ag	Antigen	kDa	Units of molecular mass in kilo Daltons
AP	Allergic patient	L	<i>Lactobacillus</i>
AFM	Atomic force microscopy	LAB	Lactic acid bacteria
AGE	Advanced glycation end products	LPS	Lipopolysaccharide
ALT	Alanine transaminase (or SGPT/ALAT; liver enzyme)	LS	Light scattering
ANOVA	Analysis of variance	LTP	Lipid transfer protein
APC	Antigen-presenting cell	MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
Ara h	<i>Arachis hypogaea</i> (peanut)	MAMPs	Microbe-associated molecular patterns
ASIT	Allergen-specific immunotherapy	MHC	Major histocompatibility complex
AST	Aspartate transaminase (or SGOT/ASAT; liver enzyme)	MLN	Mesenteric lymph nodes
αCD3	Antibody directed against CD3	MMC	Mucosal mast cell
αCD28	Antibody directed against CD28	Mr	Relative molecular mass
BAT	Basophil activation test	MRA	Mediator release assay
BCA	Bicinchoninic assay	MS	Mass spectroscopy
B-cell	Lymphocyte which matures in bone marrow	Mw	Molecular weight
Bet	<i>Betula pendula/verrucosa</i> (birch)	N (n)	Native (unheated)
BHR	Basophil histamine release	NA	Non-allergic
BrdU	5-bromo-2'-deoxyuridine	NBT	Nitroblue tetrazolium
BSA	Bovine serum albumin	NK cell	Natural killer cell
CBA	Cytometric bead array	NLRs	Nucleotide oligomerisation domain(NOD)-like receptors
Ca-I	Calcium ionophore	nsLTP	Nonspecific lipid transfer proteins
CAST	Cellular antigen stimulation test	OAS	Oral allergy syndrome
CCD	Carbohydrate determinant	OD	Optical density
CD	Circular dichroism	OPA	o-Phthaldialdehyde
CDx	Cluster of differentiation x	PBMC	Peripheral blood mononuclear cells
CFSE	Carboxyfluorescein diacetate succinimidyl ester	PBS	Phosphate buffered saline
ConA	Concanavalin A	PHA	Phytohemagglutinin
Cor a	<i>Corylus avellana</i> (hazelnut)	PI	Propidium iodide
CRD	Component-resolved diagnosis/diagnostics	PMA	Phorbol myristate acetate
Da	Dalton	PR	Pathogenesis-related
DBPCFC	Double-blind, placebo-controlled food challenges	PRR	Pathogen-recognition receptor
DC	Dendritic cell	r	Recombinant
DH	Degree of hydrolysis	R	Isolated from roasted peanuts
DNA	Deoxyribonucleic acid	R+g	Heated in a dry form for 20 min at 145°C in the presence of glucose
EAST	Enzyme allergosorbent test	R-g	Heated in a dry form for 20 min at 145°C in the absence of glucose
EC50	Allergen dose inducing 50% of the max. release (half maximal effective conc.)	RAST	Radioallergosorbent test
ELISA	Enzyme-linked immunosorbent assay	RBL cells	Rat basophilic leukaemia cells
EPSB	EuroPrevall serum bank	Rpm	Rounds per minute
ESIT	Anti-IgE immunotherapy	RPMI	Roswell Park Memorial Institute medium
FACS	Fluorescence-activated cell sorter	RT	Room temperature
FCS	Fetal calf serum	SD	Standard deviation
FcεR1	High affinity receptor for IgE	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
FT-IR	Fourier transform infrared spectroscopy	SEC	Size exclusion chromatography
G	Heated in wet form for 15 min at 110/110°C in the presence of glucose	SILT	Small intestinal lymphoid tissue
GALT	Gut-associated lymphoid tissue	SLIT	Sublingual immunotherapy
GI	Gastrointestinal	SPT	Skin prick test
Gly m	<i>Glycine max</i> (soy)	Tc	Cytotoxic T cell
GGT	Gamma-glutamyl transpeptidase	T-cell	Thymus-derived lymphocyte
H	Heated in wet form for 15 min at 110/110°C in the absence of glucose	TCR	T cell receptor
HPLC	High-performance liquid chromatography	TGF-β	Transforming growth factor-beta
IC50	Concentration inhibitor (competitor) that inhibits 50% of the IgE binding to the native allergen	Th	T helper cell
IFN-γ	Interferon-γ	TLR	Toll-like receptor
Ig	Immunoglobulin	TNF-α	Tumor necrosis factor-α
IL	Interleukin	Treg cell	Regulatory T cell

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Chapter *1*

General introduction

Parts of the introduction are from Chapter 12: Influence of food processing, digestion and the food matrix on allergenicity & cellular measures of allergenicity. Yvonne M. Vissers, Harry J. Wichers and Huub F.J. Savelkoul; In: Multidisciplinary Approaches to Allergies, Edited by Zhongshan Gao, Huahao Shen, Min Zheng, Lynn J. Frewer and Luud J.W.J. Gilissen, Co-publishing by Zhejiang University Publishing and Springer, 2011 (*in press*)

Food allergy and immune cells

Adverse food reaction represents any abnormal clinical response associated with ingestion of a food or a food additive. There are two main adverse reactions to food: non-toxic and toxic (Fig. 1). The non-toxic reactions can be further classified as non-immune-mediated (food intolerance) and immune mediated (food allergy) based on the pathophysiological mechanism of the reaction. In addition, the immunological or truly allergic reactions to food can be further classified in IgE mediated (type 1 hypersensitivity) and non-IgE mediated (type III or type IV hypersensitivity) reactions [1,2]. This thesis is focussed on IgE mediated allergic reactions, in which the allergic symptoms are initiated by IgE antibodies that are produced in response to otherwise harmless environmental antigens, i.e. allergens.

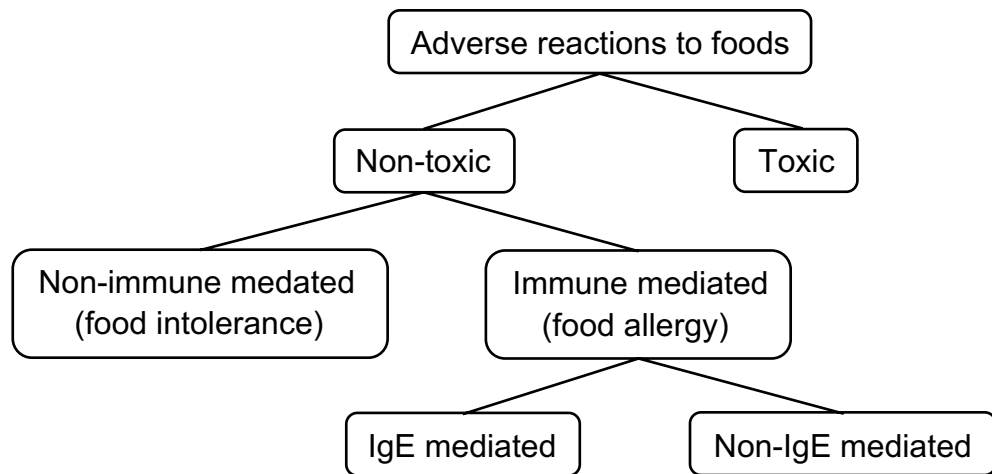


Fig. 1. Classification of adverse reactions to foods (adapted from [1,2]).

The incidence of IgE mediated allergies in most developed countries is commonly in the range of 20 to 30% of the total population, of which half may develop allergic rhinitis. This incidence has been rising since about 1960 with a possible recent plateau in developed countries, as indicated from the International Study of Asthma and Allergies in Childhood (ISAAC). This study summarized worldwide trends in the prevalence of asthma, rhinoconjunctivitis and eczema [3]. A large variation was observed among centres and increases in the prevalence of all allergic conditions in the period 1994-2003 were reported in more centres than decreases, but a tendency was found for a lack of increase in asthma and eczema in centres with previously high prevalence [3,4]. Explanations for this increase in allergy and asthma have been associated with an increased exposure to house dust mite because of modern housing, the improved hygiene and cleanliness and widespread antibiotic use, an increased prevalence of obesity and dietary changes [5]. Current allergy treatment is almost exclusively concerned with the management of allergic symptoms using drugs such as anti-histamines and steroids supplemented with, in the case of allergic asthma, drugs to improve breathing such as beta-2 agonists. Al-

though these symptomatic drugs provide temporary relief, they can be inconvenient to use and can cause unwanted side effects. For IgE mediated food allergy there is currently no other cure than complete allergen avoidance, which is often difficult to achieve and better knowledge of allergenicity of food allergens, the process of allergic sensitization and the development of clinical symptoms is urgently needed.

IgE mediated food allergy is a disease affecting all age groups. There is no well-established cure or preventative treatment for food allergy at the present time. As management is restricted to avoidance of problematic allergens, having a food allergy changes the quality of life experienced by a food allergy sufferer and their caregivers in a profoundly negative way. Food allergic adolescents and adults reported to have poorer overall health, more limitations in social activities and less vitality compared to individuals from the general population [6]. Mothers of children having a peanut allergy reported to have a significantly poorer quality of life and suffered more anxiety and stress than fathers [7]. In addition, peanut allergic subjects felt more threatened by potential hazards within their environment, felt more restricted by their peanut allergy regarding physical activities, and worried more about being away from home. They reported lower scores on quality of life than children with insulin dependent diabetes mellitus [7,8].

Sensitization and clinical symptoms

Sensitization is the initial phase in the development of IgE-mediated allergy. When an allergen enters the body via the epithelial barrier of the skin, airway or gut, it is taken up by antigen-presenting cells (APCs), such as dendritic cells (DCs). After processing of the allergen, the allergen-derived peptides are bound to major histocompatibility complex (MHC) class II molecules on the surface of APCs. In this way these peptides are presented to the T cell receptor (TCR) on naïve CD4⁺ antigen-specific T cells which triggers these cells to develop into Th1, Th2 or Th17 effector cells or regulatory T (Treg) cells. If APCs cause differentiation to Th2 cells, production of IL-4 and IL-13 from these cells will drive neighbouring B cell heavy-chain class-switching to IgE production and secretion of allergen-specific IgE antibodies. The secreted allergen-specific IgE will subsequently bind to the high affinity FcεR1 receptor of mast cells in the tissue or basophils in the blood, which completes the sensitization phase.

Upon a second exposure to the same allergen, this can bind to the cell-bound IgE and in that way cluster and cross-link two or more FcεR1 receptors on the surface of the mast cell or basophil. The number and duration of cross-links per basophil or mast cell should be at least 100 for at least 100 seconds to induce mast cell and/or basophil activation, subsequent degranulation and the release of inflammatory mediators [9] like histamine, leukotrienes, platelet-activating factor, prostaglandins and cytokines. Within minutes after the allergen exposure, histamine and leukotrienes cause increased local blood flow and vascular permeability, leading to swelling and an increased cellular infiltration, while proteases cause tissue damage and platelet-activating factor has been shown to play an important role in the induction of anaphylaxis. Subsequently, this will lead to a variety of immediate cutaneous, respiratory, and/or gastrointestinal symptoms (Fig. 2).

The release of pro-inflammatory mediators and cytokines from mast cells, and the increase in vascular permeability promotes subsequent recruitment of other effector cells, e.g. eosinophils neutrophils, basophils and Th2 lymphocytes [10]. In some patients, this can lead after

6-24 h to a late-phase, cell-mediated response frequently seen in allergic nasal, respiratory and skin disease (atopic eczema). This could convert into a chronic inflammatory response, like in chronic asthma, if antigen persists and stimulates allergen-specific Th2 cells, which in turn promote eosinophilia and further IgE production.

It is not known why some individuals start producing IgE when encountering allergens while others do not. Probably there are several factors involved, such as the host genotype, type of allergen, allergen concentration in the environment, route of exposure and whether exposure occurs together with agents that can either enhance or down-regulate the sensitization process [11]. It is also unclear what factors act to prevent the development of clinical (symptomatic) food allergy in otherwise sensitised individuals. In addition, it is only partly clear what factors determine the severity of food allergic reactions. Threshold of exposure for elicitation of a food allergic reaction during challenge of food allergic subjects, and the intensity of those reactions, differ between individuals, and vary with time in the same individual. Genetic background, medication history, disease history, the time of the day or the stage of the year of exposure to allergens, geographical parameters, variation in diet, the abundance of an allergen in the diet, the degree of digestion by the gastro-intestinal tract, the degree of processing, and the impact of embedding in food matrix components are all conditions that can influence this threshold and the severity of the allergic reactions [12,13].

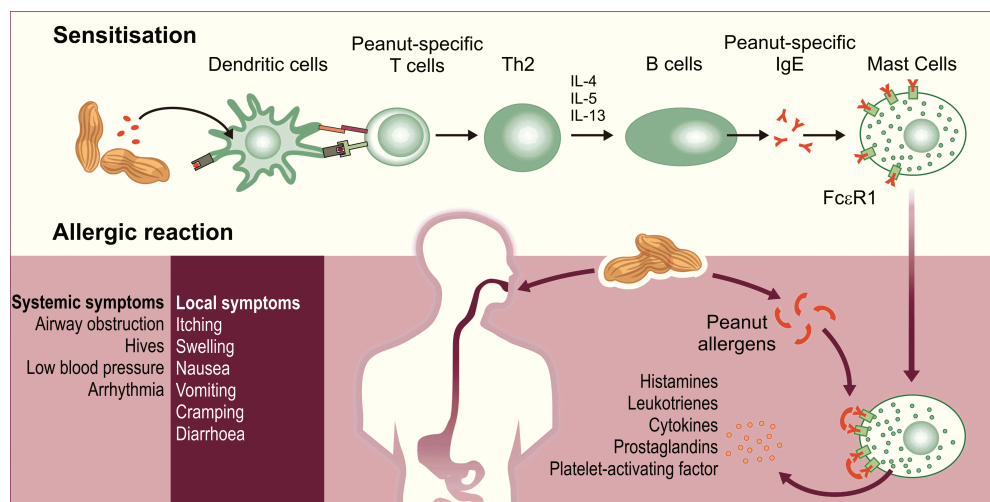


Fig. 2. Sensitization and elicitation phase of the allergic reaction (adapted from [14]).

Peripheral blood mononuclear cells (PBMCs)

The cells in human blood consists of erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (blood platelets). The leukocytes part (with a normal range of $4.0 - 11.0 \times 10^9/L$) is made up of granulocytes (neutrophils (54-62%), eosinophils (1-6%) and basophils (<1%)), monocytes (2-10%, with a normal range of $0.2 - 0.8 \times 10^9/L$) and lymphocytes (25-33%, with a normal range of $1.5 - 4.5 \times 10^9/L$ consisting of T cells, B cells and natural killer [NK] cells). The monocytes and lymphocytes together form the peripheral blood mononuclear

cell fraction (PBMC). PBMC can be isolated from the peripheral blood by density gradient separation and these cells can be used to assess cell-mediated immunity in general or, via antigen-specific stimulation, to detect previous exposure to a variety of antigens/allergens and to monitor for example the response to immunotherapy.

T cells and antigen-presenting cells (APCs)

In the blood, 60-70% of T cells (normal range of CD3+ T-cells is $0.69 - 2.54 \times 10^9/L$) are CD4+ (normal range is $0.41 - 1.59 \times 10^9/L$) and 30-40% express CD8+ (normal range is $0.19 - 1.14 \times 10^9/L$). CD4+ T cells are generally designated helper cells and activate both humoral immune responses (B-cell help) and cellular responses (delayed-type hypersensitivity responses and others). CD8+ cells show a major cytotoxic activity against cells infected with intracellular microbes, and against tumour cells. In the blood of healthy controls activated CD3+CD25+ T-cells are present with a normal range of $<0.1 - 0.4 \times 10^9/L$. A portion of the circulating CD4+ cells play an important regulatory role that acts to down modulate immune responses. These regulatory T (Treg) cells consist of natural occurring Treg cells (CD4+CD25+ Treg) and adaptive or induced Treg cells (Tr1). Treg cells are able to inhibit the development of allergen-specific Th2 and Th1 cell responses and therefore play an important role in a healthy immune response to allergens [15].

Both CD4+ and CD8+ T cells differentiate into functionally distinct subsets after exposure to antigenic peptides processed and presented by antigen presenting cells (APCs), like dendritic cells, B cells and monocytes/macrophages. This is best described for the transition of CD4+ T cells from naïve to effector populations. Resting naïve CD4+ T cells (designated Th cells) release very low levels of cytokines. Soon after stimulation by antigen and APC, the Th cells begin to produce IL-2 and are designated Th0. As the Th cells continue to respond to the activating signal, they differentiate to designated Th1, Th2, and Th17 cells depending on the local cytokine micro-environment at the site of activation [16]. IL-12 produced by macrophages or NK cells induces differentiation toward Th1; IL-4 produced by NK1.1+ T cells, basophils, or mast cells induces differentiation toward Th2; and TGF- β produced by Foxp3+ regulatory T-cells and IL-6 from innate immune cells or IL-21 produced by T cells and NKT cells induce differentiation toward Th17. Th1 cells are characterized by the production of IL-2, IFN- γ , and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF) and Th17 cells produce the cytokines IL-6 and IL-17 [17,18]. These effector cytokines that are produced can potentially feedback to amplify Th1, Th2 and Th17 cells and further enhance differentiation of the respective T cell subset.

The differentiation factors (TGF- β plus IL-6 or IL-21), the growth and stabilization factor (IL-23) primarily secreted by activated DCs, monocytes and macrophages, and the transcription factors (STAT3, ROR γ t, and ROR α) involved in the development of Th17 cells have been identified. The participation of transforming growth factor- β (TGF- β) in the differentiation of Th17 cells places the Th17 lineage in close relationship with CD4+CD25+Foxp3+ Treg cells, as TGF- β also induces differentiation of naïve T cells into Foxp3+ Treg cells in the peripheral immune compartment [19]. Recently, a new population of T helper cells separate from Th2 have been distinguished that produces IL-9 in large quantities ('Th9' cells). Under certain conditions relevant to chronic disease (through combined actions of IL-4 and TGF- β), these 'Th9' helper T cells seem to lack suppressive function, promote tissue inflammation and may regulate chronic

allergic inflammation [20-22].

In T-cell polarisation assays based on human PBMC-cultures, blood is obtained from allergic persons and healthy controls and PBMC are isolated by means of Ficoll separation. After performing density gradient separation, the upper fraction consists of plasma and thrombocytes, the middle fraction is the PBMC fraction, the lower fraction consists of Ficoll and granulocytes and the pellet of the erythrocytes. Because antigen-specific cultures require the presence of APCs, PBMC providing autologous APCs, instead of purified T cells are a preferred model to use. Antigen presentation in *in vitro* PBMC cultures is mainly accounted for by monocytes. The PBMC consisting of T and B cells, NK cells and monocytes are cultured and stimulated with the allergen of interest. In the cultures the allergens are presented by APCs to the adaptive immune cells via presentation of small peptide fragments of the allergens (T-cell epitopes) on the surface of the APCs. T-cell activation requires at least two signals. The MHC-peptide complex associating with the T-cell receptor (TCR/CD3) provides signal 1, whereas signal 2 is delivered via the binding of CD28 to B7-1/2 (CD80/CD86) expressed on antigen presenting cells (also called co-stimulatory interaction). Subsequently, an immunological synapse is formed, resulting in the activation of several tyrosine kinases and recruitment of adapter proteins and specific downstream signalling leading to T-cell activation and specific cytokine production [23].

Flow cytometry provides the cornerstone of cellular immunological assays and is dependent on the availability of monoclonal antibody reagents reactive with human surface and intracellular antigens. The semi-automated, rapid, accurate and large cell number measurements of cells, comprise major advantages of flow cytometric analysis. By using combinations of surface marker sets and performing multicolour flow cytometric stainings, the phenotype of the cells in culture can be analysed. A control which allows correction for possible differences between individual donors regarding proliferation or cytokine production is to perform a surface marker staining of the PBMC fraction immediately after isolation of the PBMC (Fig. 3).

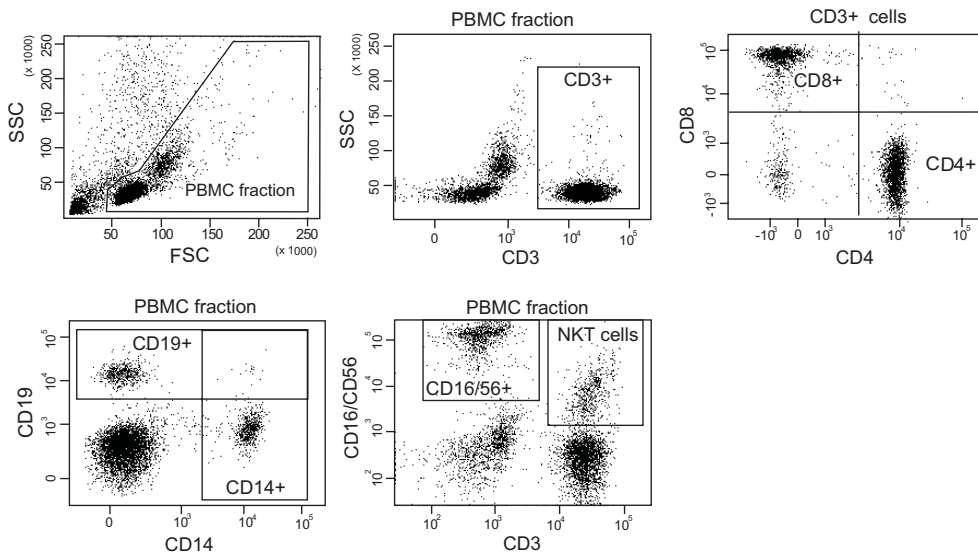


Fig. 3. (previous page) Typical data of freshly isolated human peripheral blood mononuclear cells (hPBMC) isolated from a healthy donor and stained directly with a mixture of anti (α -hCD3 (T cells), α -hCD4 (T helper cells), α -hCD8 (cytotoxic T cells), α -hCD25 (activated cells), α -hCD16/ α -hCD56 (NK cells), α -hCD14 (monocytes) and α -hCD19 (B cells). First the PBMC fraction was gated (debris was gated out) and within this PBMC fraction the percentages of T cells (65 \pm 4%), NK cells (10 \pm 3%), NKT cells (natural killer T cells) (4 \pm 3%), monocytes (12 \pm 4%) and B cells (8 \pm 2%) were determined. Within the T-cell fraction the percentage T helper cells (61 \pm 10%) and cytotoxic T cells (32 \pm 11%) were assessed. As virtually no activated cells (CD25+) were present, this result is not shown. Average \pm SD values were obtained from the average values of PBMC stained directly after isolation from the blood of 10 healthy controls. Seven-colour flowcytometric acquisition was performed on a FACSCanto II (BD Biosciences), using the BD FACS-Diva software. Per sample 10.000 events in the PBMC fraction were acquired. The following monoclonal antibody mixture was used: α -hCD3 (V450), α -hCD4 (PerCP Cy5.5), α -hCD8 (PE-Cy7), α -hCD25 (APC-H7) α -hCD14 (APC), α -hCD16 (PE), α -hCD19 (FITC) and α -hCD56 (PE).

Mast cells and basophils

Mast cells are tissue-based inflammatory cells, located primarily in association with blood vessels and in mucosal tissues lined by epithelial surfaces in the respiratory system, gastro-intestinal system, and skin, where they also mature. Mast cells that complete their differentiation in the skin or in the intestine develop into connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs), respectively. Mast cells express the high-affinity type IgE receptor, Fc ϵ R1 which binds IgE with a K_D of around 10^{-10} M. The density of human basophil Fc ϵ R1 expression correlates directly with serum IgE levels, where binding of IgE stabilizes the receptor at the cell surface. Fc ϵ R1 not occupied by IgE has a half-life on the mast cell surface of 24 hours *in vitro*, whereas receptors bound to IgE appear to be expressed for the life of the cell (around 14 days). In addition, the presence of IL-3 slows degradation of internalized receptor and some receptors appear to re-cycle to the cell surface. Mediators produced by mast cells can be divided in preformed mediators (i.e. histamine, tryptase, chymase), newly synthesized lipid mediators, and cytokines/chemokines. Collectively, these mediators are associated with the immediate hypersensitivity reaction and with the often occurring late-phase reaction occurring 6-24 h after allergen exposure. Newly released histamine has a half-life of around 1 minute in the extracellular fluid [24].

Circulating basophils share many features with mast cells, including expression of Fc ϵ R1, secretion of Th2 cytokines, metachromatic staining, and the release of histamine after IgE-mediated activation. A notable feature of basophils is their rapid and strong production of IL-4 and IL-13 when stimulated with cross-linking of Fc ϵ R1 and IL-3 [24]. Basophils also express Toll-like receptor (TLR)1, TLR2, TLR4, and TLR6 and produce Th2 cytokines in response to IL-3 plus corresponding TLR ligands [25]. Basophils are 5 to 8 μ m in diameter, exhibit a segmented condensed nucleus and contain fewer but larger granules compared to those seen in mast cells. Basophils develop from CD34+ progenitors, differentiate and mature in the bone marrow, and circulate in the periphery, where they account for less than 1% of blood leukocytes (normal range 0.01 - 0.2 $\times 10^9$ /L) and are thought to have a half-life of a few days. Basophils express a complete Fc ϵ R1, the surface expression of which directly correlates with free IgE concentration, as noted for mast cells. Aggregation of Fc ϵ R1 bound to IgE by multivalent antigen leads to basophil activation, granule exocytosis, and mediator release [24]. In addition, the proposed role of the basophil in functioning as professional APC promoting Th2 differentiation is still under

debate [25].

Even though basophils differ from mast cells in some aspects, in a clinical setting, basophils are a practical and convenient surrogate for mast cells when diagnosing allergen sensitization in patients for the practical reason that circulating basophils are much more accessible than tissue-resident mast cells [26].

Prevalence of allergy and EuroPrevall

Prevalence data

The true prevalence of food allergies is still being assessed, but is recently estimated to be around 2-5% in the US population, [27,28]. The determination of accurate food allergy prevalence rates is hampered by the lack of studies applying reliable diagnostic methodologies, such as double blind placebo controlled food challenges (DBPCFC), to large unselected populations. New data on prevalence, risk factors, quality-of-life and costs of food allergies obtained from the multi-centre birth cohort study within the EuroPrevall project (see next paragraph), is currently being analysed which may provide new insights regarding the true prevalence of food allergy [29,30].

Recent studies showed a prevalence of peanut and tree nut allergy of 1-2% in the US and Canadian population [31,32]. In some studies the prevalence was found to be much higher, with approximately 10% of 8-year-old children in the UK being sensitised to peanut, of whom 2% have a clinical peanut allergy [33]. In addition, the prevalence of peanut and tree nut allergy in children appears to be increasing [31], which contributes to a growing global concern, particularly given the severity of the allergic reactions. Furthermore, although patients with cow's milk or egg allergies have a high chance of eventually tolerating these foods or 'outgrowing' their allergy, peanut allergy tends to persist throughout life. Of the more than 3 million Americans with a peanut or tree nut allergy [31], fewer than 20% will "outgrow" the allergy naturally [34,35].

EuroPrevall

In 2005 a large EU-funded project on food allergy started named EuroPrevall (www.europrevall.org), which aimed to gain insight into the prevalence of food allergies across Europe [36]. To provide accurate information on the prevalence of food allergies, in particular standardisation of test protocols is needed. This implies also more knowledge on allergens and allergies is needed, and on the influence of food processing, digestion and the food matrix on allergenicity of individual allergens i.e. both on their potential to sensitise atopic individuals and to elicit clinical manifestations in already sensitised individuals. More knowledge on the influences of food processing and the matrix on clinical reactivity of components consumed by diet is of great importance to establish authentic standardized food materials for DBPCFC [37], which can then be used as a reference method for confirming clinical diagnosis of food allergy. This would provide, as far as possible, a concordance of clinical challenge results across the clinical centres involved in the EuroPrevall project.

More knowledge on the influence of processing is also highly relevant to the development of *in vitro* methods for diagnosing allergy since many of the foods that are consumed are ther-

mally processed and hence the original sensitising agent may be a modified, not native, allergen. Such information on the nature of such neo-allergens is also highly relevant to the development of effective *in vitro* component-resolved diagnostic methods. In addition, data on processing may allow the allergen content to be reduced in highly refined derived products below any established thresholds. This might thus provide justified criteria for exemption to the labelling regulation with no risk for allergic consumers.

Results obtained within the EuroPrevall project on the influence of processing on allergenicity of individual allergens are partly presented in this thesis.

Allergens

Food allergens are named by the first three letters of the taxonomic name of the genus of its botanical source, followed by the first letter of the species, and an Arabic number to indicate the chronology of allergen discovery and purification. As example, the first described allergen of peanut (*Arachis hypogaea*) is named, Ara h 1.

Although the diversity of the human diet is enormous, only a limited number of foods account for the majority of food allergies, with shellfish, peanut and tree nut allergies predominating in adults, whereas cow's milk, hen's egg and peanut are important in infants [28]. In addition, most allergens can only be found in a very restricted number of protein families which strongly suggests the existence of properties that would render a protein allergenic [38]. Major class I food allergens characterized to date share a number of common features; they are mostly proteins and many of these are water-soluble glycoproteins, 10 to 70 kD in size, overall highly abundant in the food, and relatively stable to heat and acid conditions, and to the presence of proteases. A protein that is stable to digestive enzymes and to processing conditions and that is abundant in the food or consumed in large amounts, is more likely to survive gastrointestinal digestion, and pass the mucosal barriers having intact epitopes that can subsequently induce an allergic reaction. However, it is clear that additional features, such as food preparation and factors derived from allergen carriers (e.g. the food matrix, microbial molecules from house dust mite and pollen grains), and the environment, can also affect the allergenicity of the compound [28,39], and no single characteristic shared by all known food allergens has been identified so far that determine or predict allergenicity of an individual protein.

In contrast to class I food allergens, class II allergens are considered to be more sensitive to heat and digestive enzymes and, therefore, cannot cause sensitizations via the oral route. Instead, they provoke allergic reactions in already sensitized patients by means of IgE cross-reactivity between sensitizing aeroallergens and symptom-eliciting food allergens leading to pollinosis-associated food allergies [40]. A well-known example is the widespread occurrence of apple allergy in North-West Europe, in birch sensitized individuals. Moreover, the different properties of class I and II food allergens also determine the clinical manifestation of food allergy. For example, allergy to fresh fruits in North-West Europe is generally associated with mild local reactions of the oral cavity, the so-called oral allergy syndrome (OAS), whereas allergy to peanuts is considered a major cause of severe anaphylaxis.

Epitopes

Binding of IgE antibodies to specific regions of allergens, known as antigenic determinants or epitopes, is a prerequisite for triggering of IgE-mediated allergic reactions. In order to bind two or more IgE molecules that can cross-link two or more FcεR1 receptors, an allergen must contain at least two IgE binding epitopes or should occur as a dimer or oligomer. Such IgE binding epitopes, also called B-cell epitopes, are frequently categorized as conformational or discontinuous epitopes or as linear, continuous or sequential epitopes (Fig. 4).

In conformational epitopes, the amino acid residues recognized by the binding site of antibodies are distributed discontinuously over the protein sequence and are brought together by folding of the polypeptide chain. Their antigenic reactivity depends on the native conformation of the protein. In linear epitopes, the amino acid residues recognized by the antibody are typically derived from short contiguous 8-10 amino acid segments which are often situated on a 30-50 amino acid stretch. However, even a sequential epitope adopts, at least transiently, a defined configuration upon interaction with an antibody complementarity-determining region which is the portion of the variable light and heavy chains that interact with the target molecule [41,42]. The conformational epitopes are expected to be more susceptible to processing-induced structural changes while the linear epitopes, are likely to be more resistant to physical treatments, and thus suggested to be more important in food allergens to cause systemic reactions. However, also linear epitopes could still be disrupted by hydrolysis and (digestive) enzymes. Conformational epitopes are important for allergens involved in OAS and respiratory allergy [43].

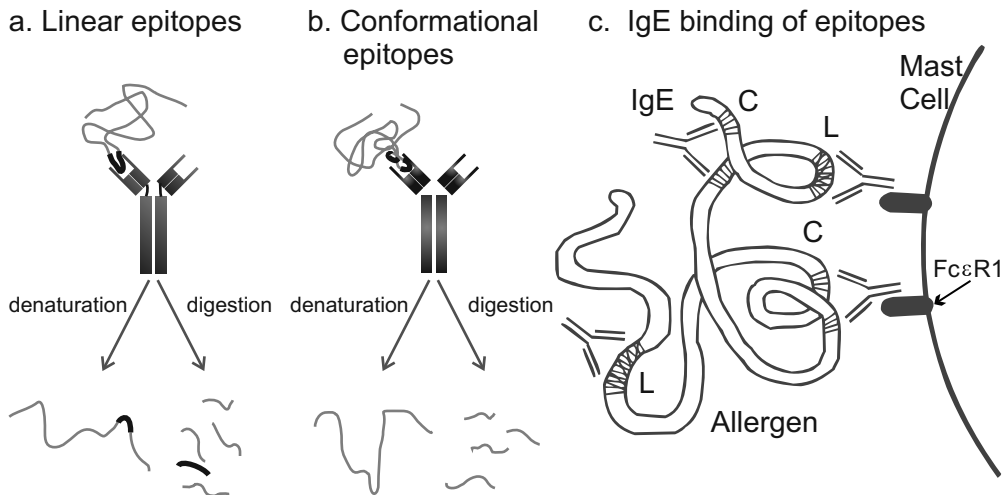


Fig. 4. Schematic representation of two antibodies interacting with linear and conformational epitopes. (a) Linear epitopes are continuous and may still be able to bind the antibody after denaturation and/or digestion (b) Conformational epitopes are discontinuously over the protein sequence and can no longer bind the antibody after denaturation and/or digestion (Adapted from www.rostlab.org/services/epitome/background.html). (c) Both linear (L) and conformational (C) epitopes recognized by different IgE antibodies bound to the FcεR1 on the mast cell (Fig. 4c: Adapted from Thesis Tiiu Saarna, 2009).

The procedures to identify conformational epitopes are technically challenging; therefore, conformational epitopes are less well studied compared to linear epitopes, even though conformational epitopes have also shown to contribute to the allergenicity of e.g. tree nut [44] and peanut [41] allergens. In addition, it has been suggested that a differential responsiveness to linear versus conformational epitopes in allergic patients could be used to better predict the severity and/or persistence of food allergies such as milk allergy [45,46].

Furthermore, allergens contain multiple T-cell epitopes, i.e. the small proteolytically cleaved linear peptides that interact with the receptors of T cells. Protein uptake by professional antigen-presenting cells through endocytosis or phagocytosis leads to formation of endosomes, which become increasingly acidic as they progress and eventually fuse with lysosomes containing MHC class II molecules. These vesicles contain aspartic and cysteine proteases which degrade the protein into peptides. The activities and specificities of the proteases in the MHC class II presentation pathway are poorly characterized. The core binding motif of both MHC I and MHC II is approximately nine amino acids long. The binding groove in MHC class II molecules has open ends, and therefore accommodates much longer peptides, possibly even whole, unfolded proteins [47-49], but typically 12-15 amino acid residues in length [50].

Respectively, 23 and 10 distinct IgE-binding epitopes were recognized along the primary amino acid sequence of Ara h 1 and Ara h 2 [51]. Five major epitopes of Ara h 1 that bound peanut-specific serum IgE from more than 60% of patients tested were identified, and were located at AA 21-34, 89-98, 393-403, 498-507, and 594-605 [52], while peptides located at AA 25-34, 65-74, 89-98 and 498-507 showed to contain immunodominant epitopes by Burks *et al.* [53]. Moreover, the epitopes 498-507 and 594-605, which were located in the C-terminal end of the protein, were in a region that shares significant sequence similarity with vicilins from other legumes [53]. In addition, 9 out of 23 sequential B-cell epitopes identified in the N-terminal part of the Ara h 1 monomer are specific for Ara h 1, while the remaining 14 characterized in the C-terminal region of Ara h 1 shared high degree of homology and conformation with other legume vicilins [54]. This may explain the possible cross-reacting antibodies to other legumes that can be found in the sera of peanut allergic patients. Immunodominant regions for serum IgE-binding for Ara h 2 have been identified at AA 27-36, 57-66 and 65-74 [55].

Data for T-cell epitopes of the peanut allergens are limited. Initial studies have identified two highly immunogenic T-cell reactive regions of Ara h 2 located at AA 19-47 and 73-119 [56]. Three major and two minor T-cell epitopes were predicted for Ara h 1 [54].

Classification of plant food allergens into superfamilies

Plant food allergens can be classified into families and superfamilies based on their structural and functional properties. The most widespread groups of plant proteins that contain allergens are the cupin superfamily (7S and 11S seed storage proteins), the prolamin superfamily (2S albumins, nonspecific lipid transfer proteins [nsLTPs], α -amylase/trypsin inhibitors, and prolamin storage proteins of cereals) and the protein families of the plant defence system (e.g. pathogenesis-related [PR] proteins, certain proteases and protease inhibitors) [57]. Fig. 5 shows a classification of 7S and 11S globulins and 2S albumins from peanut, hazelnut and soy allergens in their respective (super)families.

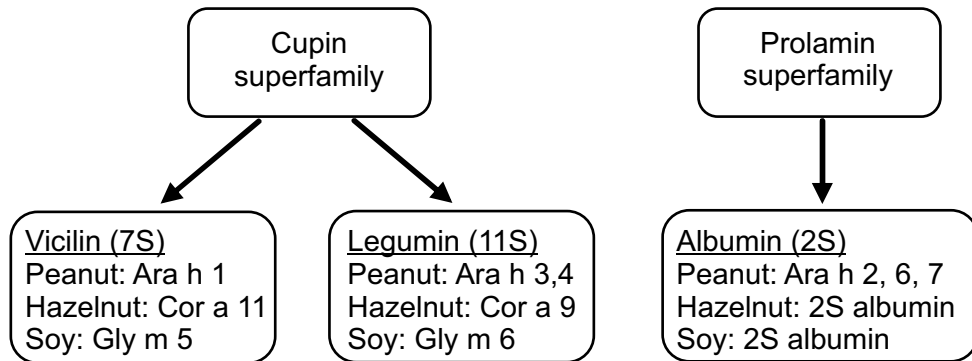


Fig. 5. Classification of 7S, 11S and 2S peanut, hazelnut and soy allergens in their respective (super)families.

Peanut allergens

Peanuts are known to cause the largest number of cases of severe anaphylaxis and deaths in the US [28]. Peanuts consist of approximately 45-50% oil, 25-30% protein, 8-12% carbohydrates, 5% water, 3% fibre and 2.5% ash [58]. Until now, 11 peanut allergens have been identified and accepted by the Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS), termed Ara h 1 to Ara h 11 (see Table 1). Eighty to ninety percent of peanut proteins belong to the seed storage proteins of the 7S vicilin, 11S legumin or 2S conglutin type. The amounts of Ara h 1 and Ara h 2 are estimated to be 12-16% and 5.9-9.3%, respectively of the total protein extract [59]; however, this can differ per peanut variety [60,61].

The two major peanut allergens, Ara h 1 and 2, are part of the vicilin and conglutin families of storage proteins, respectively and are further described below. Ara h 3 is a glycinin seed storage protein thought to occur naturally as a hexameric structure resulting from the stacking of two similarly oriented homotrimers. It is designated both as a minor and a major allergen [51,62]. Ara h 4, previously described as a distinct peanut allergen, is found to be a nearly identical isoform of Ara h 3 and therefore Ara h 3 and Ara h 4 are denoted as Ara h 3/4 in table 1 [63].

Ara h 6 and Ara h 7 are very similar to Ara h 2 and also further described below. Two of the 11 identified peanut allergens, Ara h 5 and Ara h 8, are proteins associated with pollen-related food allergy (class II allergens) and mostly associated with mild symptoms like OAS. Ara h 5 belongs to the profilin family, a group of actin-binding proteins responsible for cytoskeleton formation in plant cells. Eight surface-exposed epitopes were predicted, of which three were found common among various profilins, which could explain their cross-reactivity [64]. Ara h 8 is a member of the PR-10 family and is known to cross-react with the major birch pollen allergen Bet v 1. Birch pollen sensitized patients often show IgE binding to Ara h 8, despite their tolerance to peanut. Therefore, Ara h 8 sensitization is mainly associated with no clinical symptoms or with OAS symptoms [65-68].

Table 1. Identified peanut allergens.

Allergen	Size (kDa)	Type of protein	# of epitopes	% IgE binding ^a	References
Ara h 1 (conarachin)	63	7S globulin (vicilin)	23	35-95	[69-72]
Ara h 2 (conglutin)	17-19	2S albumin (conglutin)	10	75-100	[73-77]
Ara h 3/4 (arachin)	60*	11S globulin (glycinin/legumin)	8	20-77	[51,62,63]
Ara h 5 (class II)	15	G-actin-binding (profilin)	8	9-18	[64,78]
Ara h 6	15	2S albumin (conglutin)	no linear	38-92	[66,76,79-81]
Ara h 7	16-17	2S albumin (conglutin)	nd	43	[66,82,83]
Ara h 8 (class II)	16	PR-10 (like Bet v 1)	nd	minor	[67,68]
Ara h 9	9-10	LTP	nd	minor	[84,85]
Ara h 10	16	oleosin	nd	minor	[86]
Ara h 11	14	oleosin	nd	minor	[86]

^aPercentage of total peanut-allergic patients sensitized to allergen; LTP: lipid transfer protein; PR: pathogenesis-related; nd: not determined; minor: less than 50% of the peanut allergic patients have IgE in their serum that binds to this allergen. *Ara h 3 consists of an acidic and basic polypeptide of 45 and 23 kDa, respectively, linked together by disulphide bonds.

Ara h 9 is a non-specific lipid transfer protein, of which the two known isoforms (Ara h 9.0101 and Ara h 9.0201) share 90% sequence identity. Sensitization in most cases is probably due to primary sensitization to peach or other LTP-containing fruits, seen mostly in Mediterranean countries, and is often associated with systemic and more severe reactions next to OAS. Ara h 9 is a minor allergen in non-Mediterranean subjects, while it is a major allergen in peanut-allergic patients from the Mediterranean area. The frequency of sensitization to the classic major peanut allergens Ara h 1, Ara h 2, and Ara h 3 in these patients is low [84,85].

Ara h 10 and 11, both oleosins, were described as candidate minor allergens from peanut, but their clinical relevance across Europe is not yet known [86].

Ara h 1

Ara h 1, also known as conarachin is a vicilin-like seed storage 7S globulin. 7S globulins consist of glycoproteins that are post-translationally N-glycosylated during their processing along the endoplasmic reticulum and the Golgi apparatus before they accumulate into the protein bodies of the ripening seeds [87]. Mature 7S globulins typically comprise of 50 to 60 kDa subunits assembled into trimeric proteins of about 150 to 190 kDa.

Ara h 1 was described as a 63.5 kDa protein, that occurs naturally in trimeric form (Fig. 6a) of approximately 180 kDa, that often aggregates forming multimers of up to 600-700 kDa [69,71,72]. It consists of 31% α -helices, 36% β -structures and 33% random coil and contains 23 linear IgE epitopes. The majority of the epitopes are located in the areas of the subunit-subunit contacts that are protected from protease degradation [57,70,88] (Fig. 6b).

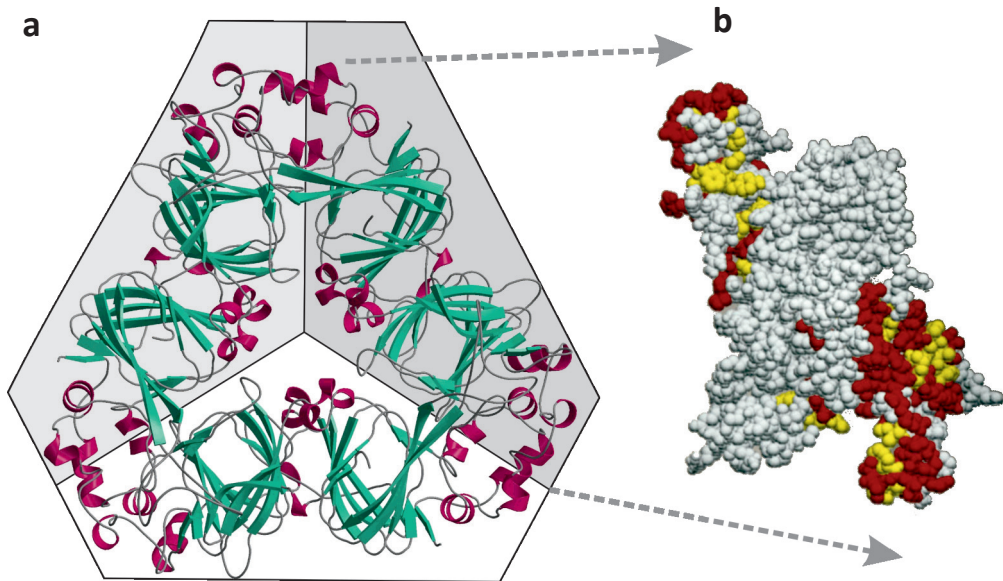


Fig. 6. (a) Ribbon diagram showing the back face of the Ara h 1 homotrimer. Strands of β -sheet and stretches of α -helix are coloured green and red, respectively. Coil structures and loops are coloured light grey (Adapted from [54]). (b) Space filling model of Ara h 1 tertiary structure. The red areas represent the IgE binding epitopes, and the yellow atoms are the residues that were determined to be critical for IgE binding to occur. The majority of the Ara h 1 IgE binding epitopes are clustered in two regions of the allergen, near the areas of monomer-monomer contact (Adapted from [71]).

Ara h 2/6/7

The abundant peanut 2S albumins Ara h 2 and Ara h 6 and the low-abundant Ara h 7 are members of the prolamin superfamily that share a characteristic cysteine skeleton with at least 8 conserved cysteine residues [83,89]. 2S albumins share a common three-dimensional structure made of 5 α -helices arranged in a right-handed super helix and connected by more or less extended loops. This conformation is maintained by 4 or 5 disulphide bridges for Ara h 2 and Ara h 6, respectively, and makes the protein structure stable to, for example, thermal processing and proteolysis [90-93].

Ara h 2, 6 and 7 comprise several isoforms of Mr 17 kDa [74,75,93], 15 kDa [79,81,93] and 16-17 kDa [83], respectively. Produced as a single chain precursor they are proteolytically processed in peanut seeds resulting in two subunits linked by intramolecular disulphide bonds [79,89]. Ara h 2 has been found to act as a weak trypsin inhibitor, that protects Ara h 1 from degradation by trypsin [94]. In addition, the low abundance of Ara h 7, might indicate a function of Ara h 7 as a novel form of trypsin or amylase inhibitors [83].

Between 75 and 100% of the peanut-allergic patients is sensitised to Ara h 2 [73,74,76,77]. Ara h 6 has also been identified as a major peanut allergen [76,79-81] whilst the allergenic peanut 2S albumin, Ara h 7 was recognised by 40-43% of peanut allergic sera [82] and has recently

been confirmed as a low abundance allergen in peanuts [66,83]. Ara h 6 and, to a lesser extent, Ara h 7 are homologous to Ara h 2 and show 59 and 35% of amino acid sequence identity, respectively [78] which may explain the high amount of cross-reactivity observed between these allergens.

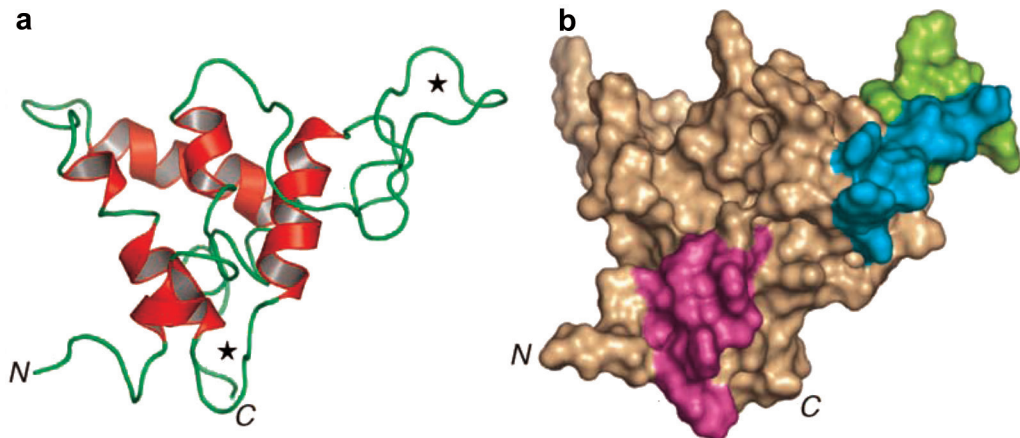


Fig. 7. (a) Ribbon diagram showing the Ara h 2 monomer. Ara h 2 differs from other 2S albumins by two additional loops located at the N- and C-terminal ends of the polypeptide chain, respectively (indicated by stars) (b) Space filling model of Ara h 2 tertiary structure. Coloured areas represent the areas occupied by 3 of 10 linear IgE binding epitopes; N-termini are oriented left (Adapted from [90]).

Cross-reactivity of peanut proteins

A cross-reaction occurs when epitopes on different allergens are structurally alike, so that an IgE antibody directed against one allergen also binds with other allergens. The degree of cross-reactivity depends among others on the extent of protein homology [95]. A high level of overall sequence homology is likely to result in IgE-binding epitopes that are of high sequence similarity, leading to cross-reactive IgE antibody interactions [96,97].

Cross-reactivity of peanut and tree nut allergens could contribute to the high incidence of tree nut sensitisation in peanut allergic individuals. Sera of peanut-allergic patients that strongly interact with Ara h 1 were shown to often react with the corresponding vicilin allergens (Cor a 11, Ana o 1, Jug r 2) from tree nuts, with Gly m 5 (β -conglycinin) from soy and with Pis s 1 from pea [87,98]. Sequence homology of 7S globulins from hazelnut (Cor a 11), soy (Gly m 5) and pea (Pis s 1) to peanut 7S (Ara h 1) are 33-34%, 40-49% and 43-52%, respectively [99].

In addition, Ara h 2 shares common IgE-binding epitopes with almond and Brazil nut allergens [97], and Ara h 3 to soybean, pea and tree nut glycinins. The glycinins in Ara h 3, soybean, and pea have a sequence similarity of 62% to 72% [100,101]. The PR-10 protein from peanut, Ara h 8, shows cross-reactivity to lupine and Gly m 4 from soybean and allergy to these proteins is likely due to primary sensitization to birch [102,103]. Ara h 9 is a lipid transfer protein (LTP) protein and sensitization in most cases is probably due to primary sensitization to peach or

other LTP-containing fruits and is most common in southern Europe [62].

Besides the serological aspect of the cross-reactivity between peanut, tree nuts, soy and lupin, not all cross-reactive antibodies give rise to clinical food allergy [104]. Usually, IgE reacts with higher affinity with the homologous epitope used for raising the antibody than with cross-reacting epitopes [42]. Clinically irrelevant positive *in vitro* test results may arise from low affinity cross-reacting IgE and therefore serological cross-reactivity is always broader than clinical cross-reactivity. The clinical relevance of cross-reacting IgE in allergic individuals still remains a puzzling question. Studying serological cross-reactivity by inhibition assays can reveal which allergen source causes sensitization, although it will not reveal which food will and which food will not give rise to clinical allergy [104]. Furthermore, it was shown that peanut cross-reacting allergens from lupine might have a higher clinical relevance compared with peanut cross-reacting allergens from other legumes [105]. Cross-reacting carbohydrate determinants (CCDs) or glyco-epitopes on structurally related vicilin allergens could also be seen as an additional source of clinically irrelevant cross-reactivity [106,107], and could result in false-positive specific IgE tests. In addition, patterns of cross-reactivity differ greatly from one patient to another [103].

In conclusion, cross-reactivity of peanut allergens to homologues proteins is common. However, as serological cross-reactivity could be clinically irrelevant, attention should be paid when evaluating IgE profiles in patients.

Diagnosis of allergy and component resolved diagnosis (CRD)

The diagnosis of food allergy can often be made or eliminated with a focused history and physical examination. If the medical history does not clearly reveal an association between a suspected food and the patient's symptoms or if it fails to identify a specific food, a diet diary may be useful. Rather than disclosing unrecognized food allergy, diet diaries often rule out allergy by revealing ingestions of the suspected food that are clearly not associated with clinical symptoms. To date, the best available test to diagnose food allergy is the double-blind, placebo-controlled food challenge (DBPCFC), which is regarded as the gold standard for food allergy diagnosis. In a DBPCFC a patient is challenged with incrementing amounts of the suspected allergenic food (verum) and with a placebo food. The verum and placebo challenges are conducted in random order and the verum is disguised (blinded) in a test food matrix. Both the patient and the physician are blinded for the sequence of the challenges. Purposes of the DBPCFC are to diagnose (the persistence of) a food allergy, to determine thresholds in food allergic patients and to gain scientific knowledge and data on food allergy. Even though the DBPCFC is the gold standard to diagnose a food allergy, it is conducted only in a limited number of centres. Reason are that the test is expensive, time-consuming, labour intensive and it also carries some risks on severe allergic reactions during the challenge. The use of standardized and validated protocols is important to perform a proper DBPCFC. Therefore specialized centres are needed to perform such tests and routine use in normal clinical settings is generally not possible. Studies are needed to develop these standardized protocols and more validated recipes, a.o. with a higher top dose than have been validated to date [108]. In addition, the added diagnostic value of the DBPCFC as compared to open food challenge tests is still under debate [109].

Sensitisation can be diagnosed *in vivo* by different skin tests and *in vitro* by different serum or plasma tests. The most commonly used skin test is the skin prick test (SPT) using food extracts and is a very sensitive test; however, it merely indicates if the patient has been sensitized to a particular food or not. There are many assays that can quantitatively measure circulating allergen-specific IgE antibodies in serum. Most common in clinical routine diagnostics worldwide are the ImmunoCAP system (Phadia AB, Uppsala, Sweden) and the Immulite system (Siemens Healthcare Diagnostics, Berlin, Germany). The ImmunoCAP is a capsule with a solid phase of a cellulose derivative to which allergens are covalently bound, while the Immulite system uses a biotinylated allergen that is bound to an avidin solid phase. IgE antibodies that react with the sample are detected by enzyme-labelled anti-IgE and a WHO reference standard for human IgE (0.35-100 kU/L) is used as a standard [110]. The different methods for diagnosis are still largely based on the use of natural total protein extracts of food products. The major disadvantages of such extracts are that these are difficult to standardize, do not allow the precise identification of the specific disease-eliciting allergens, and have, in some cases, been demonstrated to be contaminated with allergens from other sources. Well-known examples of components that could be present in the extracts and which can bind IgE, but have mostly no clinical relevance, are cross-reactive carbohydrate determinants and, to some extent, homologous cross-reactive proteins. In addition, also false-positive tests could result from testing extracts in which the disease-eliciting allergen is not present at all, or not present in a high enough quality or quantity to result in a positive test. Spiking of extracts with recombinant allergens could therefore be used to increase test sensitivity.

Single recombinant allergens or cocktails of a few defined recombinant allergens can be used in allergy diagnosis to determine a patient's individual reactivity profile, e.g. for identification of the initial sensitising allergen. Subsequently, a more appropriate form of treatment may be chosen for the individual patient as certain marker allergens could differentiate patients at risk for developing severe food allergic symptoms and patients experiencing rather mild symptoms. For this reason, such a procedure is called, component-resolved diagnosis or CRD. In addition, CRD-research will provide information on sensitization patterns in patient groups, e.g. due to geographical differences or dietary habits as recently observed for CRD of hazelnut allergy [111]. However, the specificity of the test should be investigated as well as IgE reactivity. Allergens used for CRD are mostly recombinant allergens, and thus consist of only 1 isoform, while purified native allergens often consist of a mixture of isoforms. The advantage of using recombinant allergens is that it enables production of standardized allergen preparations. However, different isoforms could differ in their allergenicity. Therefore testing for one recombinant isoform might biologically be less relevant than testing the native, purified allergen, consisting of a mixture of all isoforms. Recently, microarray chip technology has been introduced which makes it possible to test a large number of allergens with just a small amount of serum sample. This test is commercialized and available as the Immuno Solid-phase Allergen CHIP (ImmunoCAP ISAC, VBC Genomics-Phadia) It currently has 103 native/recombinant component allergens from 43 allergen sources [110]. It is important to note that, although the likelihood of clinical reactivity increases with increasing levels of food-specific IgE, the actual level often has no correlation with the severity of the reaction [112]. It will also be a new challenge for professionals involved in the care of allergic patients to properly interpret test results; clinicians should know about the basics of allergen components, their clinical implications and

should always keep in mind that sensitization does not necessarily imply a clinical allergy.

Research on CRD has increased enormously over the last years. A research from the UK including 8-year old children estimated the prevalence of clinical peanut allergy among sensitized subjects as 22.4%, while Ara h 2 was shown to be the most important predictor in clinical allergy [33]. In a population-based study, 87% of children sensitized to Ara h 1, 2 or 3 but not Ara h 8 reported symptoms after peanut intake, while of the Ara h 8 mono-sensitized persons, only 17% reported symptoms. Ara h 8 is a heat-labile protein and cooked food is therefore often tolerated. IgE analysis to peanut allergen components may be used to distinguish between peanut-sensitized individuals at risk of severe symptoms and those likely to have milder or no symptoms to peanut if sensitized to pollen allergens and their peanut homologue allergens [113].

In addition, also research on the use of recombinant peanut allergens for CRD to predict severity of the allergic symptoms has been increased over the last years. Sera from individuals with a history of severe peanut allergy, were shown to recognize more Ara h 1, Ara h 2- and Ara h 3-epitopes than sera from individuals with milder symptoms [114]. This was confirmed by a study that showed a correlation between the clinical sensitivity and the height of the polyclonal IgE response, presented by the number of epitopes recognized. No specific epitopes were associated with severe reactions to peanut [115].

Even though the DBPCFC is still regarded as the gold standard, *in vitro* diagnostic methods such as CRD could give further insight in the diagnosis and treatment of food allergy. Both CRD by measuring specific IgE using the ImmunoCAP/Immulate, as well as by microarray chip technology will be subject of further research.

Determination of allergenicity of food proteins

Antigenicity, which is a chemical property describing the ability of an epitope to react with an antibody, must be distinguished from its immunogenicity or ability of the protein to induce antibodies in a competent vertebrate host. Immunogenicity is therefore a biological property and depends on extrinsic factors such as the host immunoglobulin repertoire and self-tolerance, the induction of cytokines as well as cellular and regulatory mechanisms of the immune system. A peptide fragment showing antibody-binding, is not necessarily also able to elicit antibodies that cross-react with the protein fragment [42]. To a clinical allergist, allergenicity reflects the capacity of an allergen to induce symptoms or a skin reaction, whereas to an immunologist, it reflects either a peculiar type of immunogenicity (ie, the capacity of a protein to induce IgE antibodies) or simply the capacity to bind IgE antibodies [116]. In this dissertation we will mainly refer to immunoreactivity as the capacity of an allergen to bind IgE antibodies and to allergenicity as the capacity to cross-link IgE and thereby release mediators from basophilic granulocytes. In this thesis we did not study the capacity of proteins to sensitize and thus the capacity to induce IgE antibodies.

IgE binding

To assess the allergenicity of allergens, IgE binding studies are usually performed using methods such as enzyme immunoassays or immunoblotting. In those studies, sera from individu-

als with a known allergy that will contain allergen-specific IgE antibodies are determined by binding to a solid phase-bound allergen. These assays have a few drawbacks. Interactions with other competitors such as specific IgG antibodies may impair the binding to IgE antibodies, and binding the allergens to a plate could change the folding and thus the structure of the proteins and could make certain epitopes unavailable for IgE binding. Both drawbacks could be solved by performing a reverse assay in which anti-IgE instead of the allergen is coated to the plate and a biotinylated allergen is used as tracer in solution. A reverse inhibition assay can be used to study the inhibition of the IgE binding to the allergens. Competition is conducted by adding increasing concentrations of the allergens as competitors at the same time as the allergen-tracer. Results can be expressed as B/B_0 , where B_0 and B represent the amount of labelled allergen bound to the immobilized IgE antibodies in the absence or presence of a known concentration of inhibitor, respectively. Calculated IC₅₀ values correspond to the concentrations of competitor that inhibit 50% of the IgE binding to the labelled allergen [117].

A drawback of all IgE binding assays in general is that they detect only the binding capacity of the allergen to IgE and do not determine whether the allergen can cause cross-linking of FcεR1 bound IgE antibodies on a basophil or mast cell. This is crucial since such interactions determine the ability of proteins to trigger degranulation of effector cells, resulting in mediator release and finally in clinical manifestations of the allergic reaction. More than one epitope or IgE binding site is required to cause IgE cross-linking and an allergen with a single IgE binding site must be bound or cross-linked to another molecule with another or similar IgE binding site in order to cause histamine release. Therefore, the biological activity of proteins cannot be assessed by IgE binding tests such as an ELISA alone, and cellular assays, in which cross-linking is required to provoke a response, are needed for the proper determination of the elicitation potential of allergens.

In vitro cellular tests such as histamine release tests using autologous or passively sensitized heterologous human basophils, or the cellular allergen stimulation tests (CAST) measuring sulfidoleukotriene release from basophils, can assess the ability of proteins (allergens) to degranulate basophils from human blood. An alternative readout of allergenic activity is to analyse upregulation of the basophil-specific activation markers CD63 or CD203c by flow cytometry, which is called the basophil activation test (BAT). BAT and CAST assess the biological function of the observed IgE reactivity, this in contrast to serological assays which solely indicate sensitization rather than clinically relevant IgE reactivity resulting in food allergy. Examples where BAT and CAST are used in the diagnostic process of several IgE-mediated allergies are classical inhalant allergies (directed against house dust mite, cat epithelium, and pollen), hymenoptera venom allergy, natural rubber latex allergy, primary and secondary food allergies, drugs, NSAID hypersensitivity and component-resolved diagnosis [118]. The BAT and the CAST may complete the conventional SPT and specific IgE levels to discriminate between clinically relevant food-specific IgE vs. irrelevant IgE responses.

In vitro functional tests with a heterologous rat basophil cell line that is stably transfected with the genes of the human FcεR1 could be used instead of donor-derived basophils that have to be freshly analysed to study the biological activity of purified proteins or to study the effect of processing or digestion on their allergenic potential. Elicitation of an allergic reaction upon allergen exposure in recently developed animal models can be used to assess the potential of such an allergen to elicit clinical manifestations in already sensitised individuals (elicitation

potential). A comprehensive and complete overview on *in vivo* and *in vitro* techniques to determine the biological activity of food allergens can be found in the review of Poulsen [119] and more recently immunological techniques have been described by Sancho *et al.* [120]. A recent review extensively discussed physicochemical and immunological techniques to characterize plant food allergens [121].

Besides assessing IgE binding and cross-linking of allergens, another informative aspect is to determine T-cell mediated immune reactions. To assess whether the processed or digested food protein is still able to activate CD4⁺ helper T cells and skew the responding cells towards a Th2 like profile, T-cell polarisation assays can be used. Some treatments (such as cooking or *in vitro* digestion) could have a different effect on IgE binding and cross-linking compared to T-cell mediated immune responses [122,123], which makes it interesting to perform both types of assay. Below, basophil assays (basophil histamine release tests and the mediator release assay using RBL cells) and T-cell assays will be discussed more in detail.

IgE cross-linking using mediator release assays

Basophil Histamine Release test (BHR)

The principle of the basophil histamine release test is to challenge isolated and sensitized basophils with an allergen which will cross-link surface-bound specific IgE antibodies causing histamine to be released from the cells. Distinct properties of the IgE repertoire, such as total and allergen-specific IgE antibody concentration, IgE affinity, and IgE clonality, are all correlated with the extent of effector cell degranulation [124]. Histamine can be measured fluorometrically after coupling to a fluorophore (*O*-phthaldialdehyde), immunochemically (by the Immuno-tech Radioimmunoassay) or by performing an automated fluorometric histamine assay. In one application of the histamine release method, glass-fibre coated microtiter plates are used for separation of histamine from other constituents of the assay. Histamine content is determined and a dose–response curve can be constructed and be compared with an appropriate standard. The amount of released histamine can also be expressed as a percentage of total histamine content of non-challenged cells using detergent-induced release. Furthermore, besides using blood from sensitized patients, a passive sensitization method can be applied. Basophils from a non-sensitized person are used, the receptor-bound IgE which is natively present is stripped from the surface of the donor basophils, and the cells are subsequently passively sensitized with human serum containing specific and relevant IgE-antibodies [119,125-127].

Despite the widespread use, the above described methods suffer from some drawbacks. Basophils are present in blood in low numbers (less than 1% of leukocytes equalling $0.01 - 0.2 \times 10^9/L$), and they are difficult to purify [128]. Furthermore, fresh blood cells from specific allergic donors or non-allergic donors in case of passive sensitization, are needed for each experiment and it requires processing of blood samples immediately after collection, which may present logistical obstacles. Another drawback is that around 10% of basophil donors have non-responsive basophils [129,130] which makes the test less reliable in assessing the level of individual responsiveness. An option to overcome these problems is by using a stable cell line which can be passively sensitised with serum IgE from allergic individuals.

Mediator release assay using RBL cells

The RBL assay which mimics the mechanism of a type 1 allergic reaction [131] uses transfected rat basophilic leukaemia (RBL) cells that recombinantly express the human $Fc\epsilon R1$ receptor [132-137]. These cells bind human IgE antibodies and have all functional properties of mast cells and basophils. β -hexosaminidase, which is present in the granules and released together with histamine, has been chosen as a surrogate marker for histamine release. The added substrate is hydrolysed by this active enzyme, resulting in a coloured product that can be measured in a spectrophotometer. A microscopic picture of RBL cells in culture and a simplified graph of the histamine release test are depicted in Fig. 8.

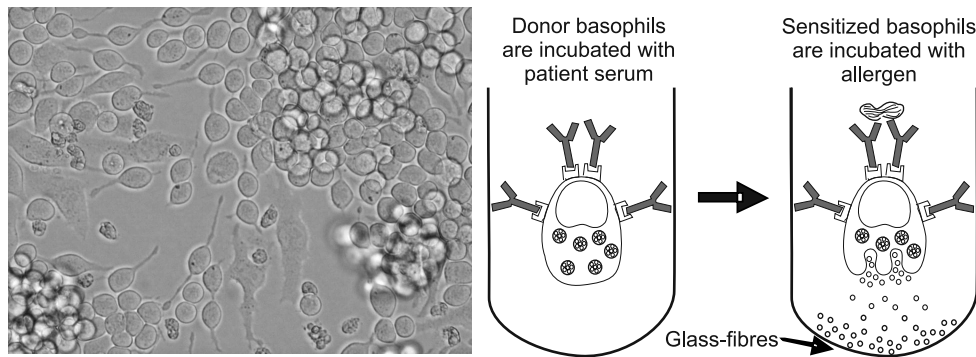


Fig. 8. (a) Microscopic picture of RBL-cells in a culture flask. Cells are not confluent yet, therefore some cells are still stretched. Furthermore it can be observed that the cells grow in layers (b) Simplified graph of the histamine release test using glass-fibre coated microtiter plates (Adapted from: Roitt, Brostoff & Male, Immunology (5th edn) Mosby, London 1998, p.301).

The mediator release assay allows, among others, the measurement of the biological activity (or potency) of allergens, the detection of allergens in various samples and the analysis of cross-reactivities between allergens. A useful indicator of the allergenic potency of allergens is the dose needed to efficiently induce cell degranulation and values corresponding to the dose of allergen that induced 50% of the maximum release (EC_{50} values). A limitation of this cell-based test is that in general only sera with higher percentages of allergen-specific versus total IgE can be used. It was found that, for sera from peanut allergic patients, the most effective sera had at least 50 kU/L of total IgE and 15 kU/L of peanut-specific IgE and contained >10% peanut-specific IgE [132]. Furthermore, the number of $Fc\epsilon R1$ on the transfected RBL cell-lines may be limiting. For example, RBL SX-38 cells have approximately 100,000 receptors/cell [138] versus 500,000 receptors/cell on basophils from atopic individuals [139]. Together, the low levels of specific IgE in the serum and low expression levels of $Fc\epsilon R1$ on the RBL cells may result in most of the IgE receptors being occupied with non-specific IgE antibodies. A number of serum factors, as well as the degree of severity of the allergic subjects clinical response have been suggested as important for inducing mediator release with these cell-lines [135]. A recent study showed that IgE affinity purification of sera increased the reproducibility and sensitivity of RBL SX-38 cells, which suggested that some factors in the serum may hamper the binding of IgE to the $Fc\epsilon R1$ [140].

T-cell polarization assays

T-cell polarization assays are used to investigate T-cell mediated reactivity towards allergens and allergenic proteins. Allergic diseases result from an abnormal T-cell response to allergens dominated by long-lived Th2 cells [141]. Allergen-specific CD4⁺ Th2 cells secrete high amounts of IL-4 and IL-13, which induce the production of allergen-specific IgE antibodies that mediate immediate allergic symptoms [124,142]. In addition to this indirect involvement in immediate reactions, allergen-specific Th2 cells have been demonstrated to be directly involved in clinical late-phase reactions in target organs such as the lung and skin [122,143].

Cytokine production

Activated (and differentiated) cells will produce a characteristic cytokine production profile. T cells of allergic individuals will typically produce IL-4, IL-5 and IL-13, which are signature Th2 type cytokines. Cytokine production can be measured in several ways. Most often the cytokine production is measured in the supernatant of the cultured cells. Cytokine enzyme-linked immunoabsorbent assay (ELISA) or flow cytometric techniques like the multiplexed bead assay (Cytometric Bead Array Flex sets, BD Biosciences) are commonly used to detect cytokines from cell supernatant or serum. Real-time quantitative polymerase chain reaction (RT-qPCR) technique can be implemented to assess mRNA expression of the cytokines in T-cell fractions. However, these methods do not allow determining which proportion of the cells is responsible for the production of specific cytokines. Multiple staining using combinations of cell surface markers and staining for intracellular cytokines permit the assessment which type of cell produces what cytokines. This approach can give valuable extra information at the single-cell level. Optimizing experiments by assessing the optimal stimulation period for the different cytokines is important as each stimulus results in different kinetics and these kinetics are crucially different for the individual cytokines. For example, monocyte derived cytokines such as TNF- α and IL-1 β were present in cell cultures already after 1 day of culture and amounts decreased significantly over time, while T-cell derived cytokines such as IL-5, IL-13 and IFN- γ showed a slower kinetics and were highest on day 7 of the culture [23].

Proliferation

This optimization is also important when measuring proliferation of cells. Proliferation of PBMC can be measured using different methods, such as incorporation of ³H-thymidine, staining with Ki-67 antibodies, loading with BrdU (5-bromo-2'-deoxyuridine), and CFSE (carboxyfluorescein diacetate succinimidyl ester) staining, each provide slightly different information. The ³H-thymidine and BrdU methods determine the S phase of the cell cycle activity by measuring the thymidine incorporation into newly synthesized DNA of replicating cells. The Ki-67 antigen however, is expressed in all active stages of the cell cycle (all phases except the G₀ phase) and cells which are still in the G₁ phase of the cell cycle will be detected by a Ki-67 staining and not by a ³H-thymidine incorporation assay or a BrdU assay. Upon cell division, the CFSE is covalently bound to free amine groups of intracellular macromolecules in the cell, is distributed uniformly between daughter cells. CFSE staining can be the method of choice especially when the rate of the divisions of the cells is studied and when working with a uniformly sized cell popula-

tion (such as resting T or B lymphocytes). However, hPBMC consist of a non-homogeneous population of cells (not uniformly sized and therefore not uniformly stained) which can make it difficult to differentiate between undivided and divided cells. As a result, this could decrease the sensitivity of the test. Furthermore, care should be taken when using CFSE because of its toxicity for dividing cells and effect on the expression of activation markers, like CD69 and CD25. A disadvantage of ^3H -thymidine incorporation is that it does not allow identification of the phenotype of the proliferating cells, as it can only measure bulk cell divisions, a limitation overcome by flow cytometry (Ki-67 and CFSE staining), which enables analysis at a single-cell level. The use of selected CD markers in the same staining will enable to specify the phenotype of proliferating cells. It is difficult to directly compare the methods as they use different detection techniques (flowcytometry using fluorochromes *versus* scintillation using radio-activity) and furthermore, they also give different types of information. Therefore, it is advisable to use the same method in all experiments to be able to compare results.

Stimuli

The frequency of an antigen-specific lymphocyte in the blood is in general low. The frequency of Bet v 1-specific CD4+ T cell was estimated to be in the range of 0.0001 to 0.001% of circulating CD4+ T cells outside the birch pollen season, for both allergic and non-allergic individuals and up to 0.1% of allergen-specific T cells for both groups during the pollen season. A significant expansion of specific T cells occurred during exposure to seasonal allergens [144,145]. The frequency of antigen-specific T cells is observed to differ per allergen. The frequency of peanut-specific circulating CD4+ precursors was found to be $0.604 \pm 0.043\%$ in peanut allergic patients and about 10 times lower in both non-allergic and peanut sensitized donors. Frequencies of house dust mite-specific T cells in the blood of patients with atopic dermatitis varied between 0.002–0.026% [146]. As a result of the low frequency of antigen-specific T cells, antigenic stimulation typically activates only a very small fraction of T cells. In mildly sensitized patients in particular, allergen-specific stimulation will reveal hardly any significant proliferation and cytokine synthesis. Therefore, amplification of the response by the use of polyclonal stimuli is often used.

As a positive control, polyclonal stimuli can be used to assess the maximum stimulation capacity of the PBMC *in vitro* and thereby assess possible differences in the intrinsic immune responsiveness of antigen-specific cell cultures of different donors. Examples of mitogens are concanavalin A (ConA), phytohemagglutinin (PHA), phorbol myristate acetate (PMA) plus calcium ionophore (Ca-I) and lipopolysaccharide (LPS). Each stimulus acts at different cell surface molecules or by direct activation of intracellular molecules and uses different signal transduction pathways to activate the cells. Anti (α)-CD3 and α CD28 have been widely used to provide all T cells with the required antigen-driven activation signals, and involves the TCR-CD3 complex thereby mimicking antigen presentation. It is therefore the most physiological way of stimulating all T cells in a polyclonal fashion to assess the maximal stimulation capacities of the PBMC of the different donors. Microscopic pictures of unstimulated and α CD3/ α CD28 stimulated cells are depicted in Fig. 9. Furthermore, α CD3/ α CD28 can also be used as *in vitro* polyclonal activation stimulus for T cells in long-period cultures to re-activate and increase the antigen-specific signal. A disadvantage is that also non-specific T cells that survived the long-term culture will react upon polyclonal activation.

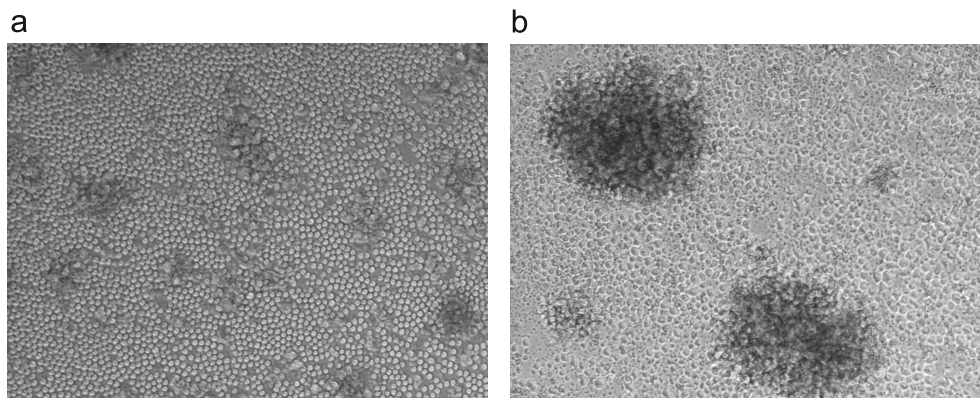


Fig. 9. Microscopic picture of (a) unstimulated cells after 4 days of culture (b) α CD3 α CD28 stimulated cells after 4 days of culture. Cell clones are clearly visible after this stimulation condition.

Factors influencing allergenicity

More knowledge on the influence of food processing, digestion and the food matrix on allergenicity of individual allergens is important in diagnostic procedures, e.g for development of *in vitro* diagnostic tests and in particular when food challenges are part of the diagnostic process. In addition, more knowledge on processing of allergens could help in modulating the allergenic properties of foods and develop desensitisation strategies, all of which may potentially reduce the disease-burden for those affected. For more information see section 'Prevalence of allergy and EuroPrevall'.

However, as a result of its complex nature, the impact of food processing and the food matrix on allergenicity of proteins has only recently become a subject of research. Such investigations are fraught with difficulties, not least the fact that food processing often renders food proteins insoluble in the simple salt solutions frequently employed in serological or clinical studies. As a consequence, our understanding of the impact of food processing on allergenicity is limited to the more soluble and extractable residues in foods, and the allergenic potential of insoluble protein complexes is virtually unstudied despite representing the vast bulk of food proteins consumed [147].

Processing

Nowadays, foods are increasingly processed, which may serve a variety of purposes. These could include improving general food qualities such as flavour, texture, taste and colour; improving food preservation and safety; enhancing suitability of food components for specific product applications; consumers convenience, pleasure and variety; and finally, to obtain or generate useful by-products and increase marketability of foods [148]. Frequently applied methods of food processing performed at home, in a restaurant or by the industry are peeling, cooking, frying, roasting, toasting, baking, cooling, freezing, drying, high pressure processing, sonication, sterilization and many other processing methods.

Food processing offers opportunities to alter the nature of epitopes of a protein, which is important for immune recognition by food component-specific antibodies. For example, three-dimensional epitope conformation may be modified as a result of protein denaturation treatments (e.g. various thermal processing treatments) leading to destruction or break up of epitopes and therefore leading to reduction in IgE binding capacity. However, denaturation treatments can also lead to generation of new epitopes, or to exposure of formerly hidden antigenic sites, resulting in an increase in IgE binding. Acid or enzyme hydrolysis of an allergenic protein may help to delete critical amino acids of an epitope. Whether caused by protein denaturation or hydrolysis, loss of epitope and ensuing loss of IgE binding may help to reduce the bioactivity of an allergen [148]. Different food processing methods may impact the allergenic potential of foods or proteins, but there is no general rule on how different allergenic foods or proteins respond to physical, chemical, or biochemical exposures during processing [149]. The effects of food processing may be governed by the molecular properties of an allergen and its interactions with food components.

Heating

Thermal processing is one of the most commonly used methods in food processing and effects of thermal processing on food protein allergenicity have therefore been reviewed in several recent articles [147,148,150-154]. Typically, loss of tertiary structure is followed by (reversible) unfolding, loss of secondary structure (55–70°C), cleavage of disulphide bonds (70–80°C), formation of new intra-/inter-molecular interactions, rearrangements of disulphide bonds (80–90°C) and the formation of aggregates (90–100°C) [155]. These modifications reflect a progressive passage to a disorganized structure with denaturation of the proteins that adopt an unfolded, random-coil conformation. The denatured molecules associate to form aggregates and then gels resulting in a modification of the surface properties and an increase in size [154].

Some plant proteins such as the Bet v 1 homologues from apple and cherry are considered to (partially) lose their allergenic activity upon food processing. This is well reflected by the fact that thermally processed fruit and vegetable products often show non- or less severe effects towards patients than the corresponding fresh plant material [156] a recombinant major allergen from peanut (*Arachis hypogaea*). Also Bohle *et al.* showed that *in vivo* ingestion of cooked foods containing birch pollen-related allergens did not induce OAS and that, in general, such foods can be consumed without difficulty because PR-10-like food proteins are easily denatured by thermal processing. However, *in vivo* ingestion of these birch pollen-related allergen containing foods could cause atopic eczema to worsen. In a study it was shown that 60 minutes cooking of the different recombinant Bet v 1-related allergens completely abolished IgE binding, but no reduction of the capacity to activate allergen-specific T-cells was observed [122]. Other commonly plant-derived ingredients, such as soya isolates, often comprise 11S and 7S seed storage globulins, which are relatively thermostable. 7S globulins have their major thermal transition at around 70–75°C, whilst 11S globulins unfold at temperatures above 94°C, as determined by differential scanning calorimetry. However, even upon heating to such temperatures these proteins only appear to unfold partially, while undergoing only minor conformational changes that suggest that the β -barrel motif, characteristic of these proteins, is a highly stable structure [147,157].

Maillard reaction

One of the most important chemical modifications occurring in foods during thermal processing is the Maillard reaction, which involves the reaction of free amino groups on proteins (generally lysine residues) with reducing sugars. The extent of glycation depends on different environmental conditions such as temperature, pH, water activity, duration of the heating and the concentration of the reducing sugars present [158]. The Maillard reaction may play a possible role in the allergenicity of foods as shown in several studies [156,159-166]. As mentioned before, the impact of processing methods may be different from food to food or protein to protein. Depending on the studied protein or food and the processing method applied, heating may have no effect or it may decrease or increase allergenicity. The existence of sequential and conformational epitopes, the demasking of new epitopes or the modification of epitopes through Maillard reactions can explain some of the results reported in the literature.

The type of allergen, type of food structure, and type of thermal processing may be of great importance when studying the impact of processing on food allergenicity. However, lack of knowledge on the influence of processing of different allergens and in different matrices makes it still difficult to predict the allergenicity of foods and provide allergic patients with appropriate advice on what is safe to consume [151].

Digestion

Due to the very acidic conditions in the stomach and the intense proteolysis occurring in the stomach and intestine, only small amounts of intact or immunologically active proteins are taken up by the gut mucosa. This suggested that food allergens are, at least partially, resistant to gastro-duodenal digestion in order to be able to sensitize the mucosal immune system. Susceptibility to digestion has therefore been considered an important biomarker for food allergy, the idea being that proteins or at least peptides of a few kDa that survive digestion are more likely to be allergens. Whilst this idea has some appeal, the evidence is rather equivocal; showing that peptide fragments of various sizes, produced during the digestion of a protein can still be immunologically active [167]. Furthermore, it is shown that some food allergens are rapidly and extensively degraded during digestion, whereas some other food proteins that are resistant to digestion are not allergenic. It is also noteworthy that a pregastric absorption also occurs, i.e. in the oral cavity, which explains the occurrence of symptoms few minutes after ingestion of food allergens [168].

Furthermore, even though the immune system is mostly exposed to digested proteins, it is to a lesser extent also exposed to intact allergens. It is assumed that a small fraction of intact dietary proteins are absorbed from the mature gut [169-172], thus leaving the option open that undigested proteins play a role in the allergic sensitisation process. A review on intestinal barrier function also summarized evidence from studies demonstrating that intestinal barrier dysfunction leading to increased intestinal permeability and exposure to intact proteins which in turn might promote sensitization and enhance the severity of food induced allergic reactions [173]. In addition, it has been widely documented both in humans and in animal models, that an increase in gastric pH (e.g. caused by antacid therapies) impedes the gastric protein digestion [174] and presumably facilitates the presentation of food peptides to intestinal T cells.

Food matrix

Few detailed reports have addressed the effects of matrices on allergenicity. Grimshaw *et al.* demonstrated a profound impact of the fat content of chocolate, in which peanut allergen was embedded, on both the clinical reactions and on the recognition of peanut allergen in ELISA-testing. Higher fat contents resulted in a lower ELISA-detection of the peanut and a larger dose of peanut protein was needed in the food challenge to cause a clinical reaction, but the reaction was more severe. It was suggested that the allergenic epitopes are concealed by the relatively high-fat food matrix, and are detected only after digestion of the fat. The allergens are thus released and absorbed more slowly than they were in a lower-fat matrix. This slower absorption may explain why the subjects did not experience the oral warning symptoms preceding the more severe symptoms. This research indicated the importance of the fat content of provocation recipes, because it can influence the clinical reaction of the provoked person [175].

Non-enzymatically active food matrix components, such as fats, may affect allergeo-reactivity of proteins. Effects of other matrix components are also obvious, such as raw-material borne proteolytic [176] and oxidative enzymes [160,177,178]. During in-plant maturation of the peanut allergen Ara h 1, for instance, an immunologically active N-terminal peptide is cleaved off [176]. For allergens belonging to the PR-10 family and the 2S-albumin family, treatment with polyphenol oxidases and/or with peroxidases decreases their IgE-reactivity, and both enzymes are ubiquitously present in plant material [160,177,178]. The mechanism that underlies these activities is as yet not fully clear; but both allergen cross-linking, or binding of phenolics to the allergens might be involved.

It can be concluded that food processing, digestion and the food matrix affect food allergenicity, and the magnitude of these effects can be examined by combining various assays.

Immunomodulation by lactic acid bacteria

Treatment and prevention of allergies

Currently, management of food allergies consists of educating the patient to avoid ingesting the responsible allergen and to initiate therapy (e.g. with injected epinephrine for anaphylaxis) in case of an unintended ingestion [28]. At the present time there is no curative therapy available once peanut or tree nut allergy has been established. However, several approaches are studied to decrease both the number of allergies and the severity of the allergic reactions.

One approach to mitigate the allergic symptoms is via modulation of the allergen itself by removing or diminishing the allergenic properties of a protein, e.g. in such a way that the IgE binding epitopes are eliminated or reduced, or reducing the size of the protein so that FcεR1 cannot longer be cross-linked, which could result in the production of hypoallergenic foods. Examples are elimination of the apple allergen Mal d 1 by RNA interference [179], transgene-induced silencing of allergens in soy [180] and peanut [181] and hypoallergenic, transgenic tomato fruits [182]. Another approach is traditional breeding as seen for the putatively hypoallergenic Santana apple cultivar [183,184]. However it should always be kept in mind that a proposed hypoallergen could still be allergenic for individual patients. In addition, hypoallergens could also be helpful to improve allergen immunotherapy (see below) as recently proposed for

hypoallergenic rBet v 1 derivatives for birch pollen immunotherapy [185], and for a hypoallergenic Fel d 1 variant which might be a promising candidate for application in immunotherapy of cat allergy [186]. These hypoallergens will have reduced allergenic capacity, but retained T-cell reactivity and thus immunogenicity. A phase I clinical safety study using modified peanut allergens is currently enrolling adult subjects with peanut allergy. In addition, modified food allergens can be combined with bacterial adjuvants (eg, heat-killed *Listeria monocytogenes* or heat killed *Escherichia coli*) to enhance the Th1-skewing effect and decrease the Th2-skewing effect. In future studies, probiotic bacteria might also be used as bacterial adjuvants to avoid the concerns of excessive Th1 stimulation by killed pathogenic bacteria [187,188].

The most commonly studied therapeutic strategy to decrease both the number of allergies and the severity of allergic reactions and that has already been studied for 100 years is the use of allergen-specific immunotherapy as a desensitizing therapy for allergic diseases. Immunotherapy (IT) is now implemented for seasonal pollen allergy, bee and wasp stings, and mite allergy. However, in the case of food allergies, further studies are required to establish the safety and efficacy of this approach and to define proper dose and length of treatment and duration of the treatment effect. Until now, this therapy in peanut-allergic subjects led to an unacceptable high rate of adverse reactions and experts in this field suggest that formal guidelines need to be established before use in a clinical setting [189-191]. A first study on sublingual immunotherapy (SLIT) treatment of peanut allergy in children showed primarily oropharyngeal side effects during dosing and could safely induce clinical desensitization in these children. Even though SLIT could possibly be a safer approach, larger studies are needed (now on-going: ClinicalTrials.gov no. NCT00580606) and long-term effects of continued peanut SLIT should be determined [192].

Several mechanisms of IT are proposed, e.g. the reduction of allergen-specific IgE production, the increase of the production of allergen-specific IgG, which can act as “blocking antibodies” and the reduction of allergen-specific inflammation in target organs. Other mechanisms could depend on modification of the phenotype of allergen-specific Th cells, e.g. stimulation of Th0/Th1 lymphocytes, rather than Th2 cells, and induction of Treg cells, which produce cytokines such as IL-10 and TGF- β . It remains unclear to what extent either altered memory T-cell responses, altered B-cell responses, or both are responsible for the long-lived antigen-specific tolerance that characterizes successful allergen immunotherapy [193,194]. In addition, the mechanism may differ depending on the allergen (bee and wasp venoms or inhalant allergens) and the route of immunisation [195]. Other therapies being evaluated to treat or prevent food allergy are anti-IgE monoclonal antibodies (e.g. omalizumab), vaccines using modified proteins or overlapping peptides (tested in murine models), conjugation of immune stimulatory sequences to allergen, plasmid DNA encoded vaccines and cytokines/anti-cytokine treatment [28,196]. A comprehensive and extensive review on future therapies for food allergies written by Nowak-Węgrzyn and Sampson is published recently [188].

Another approach and recent topic of interest is the use of food supplements, such as β -glucans, fungal immunomodulatory proteins and pro- and prebiotics, which could modulate the immune system and thereby suppress the allergic symptoms. The skewing of cytokine profiles from Th2 to Th1 is an often observed response to these food components [197]. Immune regulation and the microbiota in the intestine, the hygiene hypothesis and the mechanism and the use of probiotic bacteria to prevent or treat allergies is discussed below.

Immune regulation and the microbiota in the intestine

Epithelial cells in the gut (enteroendocrine cells, mucus producing goblet cells and enterocytes) express in a similar manner to immune cells, pattern recognition receptors (PRRs) to sense the presence of microbe-associated molecular patterns (MAMPs). These PRRs can be divided into three families: Toll-like receptors (TLR), nucleotide oligomerisation domain(NOD)-like receptors (NLRs) and retinoic acid inducible gen I (RIG-I)-like receptors (RLR). These receptors activate signalling cascades that finely tune epithelial cell production of antimicrobial products like secretory IgA and secretion of a diversity of cytokines and chemokines (such as IL-6, IL-8, IL-1 β , IL-7, IL-15 and CCL20), depending on the signals that are delivered by the microbes.

TLRs, which are the best characterized PRRs, are type I transmembrane proteins with ectodomains containing leucine-rich repeats that mediate the recognition of MAMPs; transmembrane domains; and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains required for downstream signal transduction. So far, 10 functional TLRs, TLR1-TLR10, have been identified in humans [198]. Each TLR family member is endowed with the ability to recognise a distinct class of conserved MAMPs. With respect to microbial recognition, TLR 1, 2, 4, 5, 6, and 9 signal via binding to common bacterial structures whereas TLR 3, 7 and 8 are aimed primarily at viral detection [199]. Microbe-epithelial interactions do not necessarily involve intact bacteria because diffusible compounds released from live or dead bacterial cells could also interact with the cognate PRR of the host. TLR and NLR can trigger expression of a surprising diversity of chemokines, cytokines, and effectors of innate immunity [200].

Epithelial cells can also recruit leukocytes to complement their barrier function or to participate in the activation of gut adaptive immune responses. The gut immune system is composed of the gut-associated lymphoid tissue (GALT), such as the Peyer's patches and small intestinal lymphoid tissue (SILT) in the small intestine, lymphoid aggregates in the large intestine, and diffusely spread lymphoid cells and plasma cells in the lamina propria of the gut. These immune cells are in contact with the rest of the immune system via local mesenteric lymph nodes (MLN). Even though the development of the GALT is initiated before birth, GALT maturation and recruitment of IgA-secreting plasma cells and activated T cells to mucosal sites only occurs after birth and is strictly dependent on microbiota-derived signals. These signals influence the crosstalk between epithelial cells and gut dendritic cells (DCs), thereby modulating the nature and intensity of intestinal B and T cell responses. It is increasingly clear how these adaptive immune elements cooperate with innate immune cells to strengthen the gut barrier and protect the host from invading pathogens [201].

The intestine is an open ecological system that is colonized immediately after birth by a microbial population that reaches an impressive amount of $\sim 1 \times 10^{14}$ commensal bacteria [202], 70-80% of which cannot yet be cultured [203]. In addition, each individual harbours at least 160 different prevalent bacterial species [202]. The number of bacterial cells present in the mammalian gut shows a continuum that goes from 10^1 to 10^3 bacteria per gram of contents in the stomach and duodenum, progressing to 10^4 to 10^7 bacteria per gram in the jejunum and ileum and culminating in 10^{11} to 10^{12} cells per gram in the colon, which contributes to 60% of faecal mass [204]. The intestinal microbiota is derived at least in part from the mother during birth and is modified thereafter by factors such as diet, antibiotic use, host genetics and other environmental factors. These commensal bacteria accomplish many beneficial functions, such as vitamin synthesis, the digestion of dietary fibre and the regulation of inflammatory

responses. The bacterial flora differs between individuals but mainly belong to two bacterial phylotypes, *Firmicutes* spp. (most notably *Clostridium* spp., *Enterococcus* spp. and *Lactobacillus* spp.) and *Bacteroidetes* spp. [203,205].

The microbiota might influence the balance between pro-inflammatory and regulatory host responses and alterations in the composition of the microbiota could jeopardize host immune responses and promote the development of various inflammatory disorders. Important is to define how individual members of the microbiota or how microbiota-derived products can affect the balance between pro-inflammatory and regulatory immune responses and to establish whether the composition of the microbiota can influence the development of inflammatory diseases in and beyond the gut [201].

Hygiene hypothesis

The 'hygiene hypothesis' first proposed by Strachan in 1989 [206], offers an explanation for the correlation between improved living conditions, higher personal hygiene and fewer siblings associated with life in the industrialized world, and increased incidence of asthma and allergies. It suggests that infections and unhygienic contact may confer protection from the development of allergic illnesses [206,207]. Even though next to allergies and asthma, a variety of auto-immune disorders have now been linked to the hypothesis [208], the hygiene hypothesis is still under investigation, and also other factors e.g. the diet can be of importance [205]. The hygiene hypothesis is based upon epidemiological data, particularly migration studies, showing that subjects migrating from a low-incidence to a high incidence country acquire the immune disorders with a high incidence at the first generation [209]. However this correlation between change in lifestyle and modifications of the incidence of immune disorders does not necessarily prove a causal relationship. In addition, the existence of conflicting epidemiological evidence would speculate that only specific infectious agents are responsible for a protective influence against atopic and autoimmune diseases [210]. Underlying mechanisms of the hygiene hypothesis are multiple and complex. They include decreased homeostasis of the immune system by a decreased antigenic competition and a decreased immunoregulation, involving various regulatory T-cell subsets and Toll-like receptor stimulation [211]. In a recent review on the mechanism of the protective effect of living on a farm for developing asthma and allergy it was postulated that activation and modulation of innate and adaptive immune responses by intense microbial exposures and possibly xenogeneic signals delivered before or soon after birth was held responsible for the decreased risk of asthma and hay fever [212].

In addition, several studies have reported differences in the composition of the faecal microbiota of infants who develop an allergic disease and those who do not. Notably, a decreased frequency of *Lactobacillus* and *Bifidobacterium* spp. has been suggested to precede the onset of allergy [213], and prophylactic approaches, which are based on the administration of probiotics to mothers and newborns at high risk for IgE-associated allergies, have been initiated.

Probiotic bacteria

Probiotics are live microorganisms, such as lactic acid bacteria, that confer a health benefit on the host when administered in adequate amounts [214]. Probiotics may alleviate inflammatory bowel disease and irritable bowel syndrome, reduce infectious diarrhoea (acute and antibiotic associated), inhibit *Salmonella* and *Helicobacter pylori* infection, influence metabolism of di-

etary compounds in the gut lumen (e.g. lactose digestion and lipid and oxalate metabolism) and secrete enzymes and bacteriocins into the gastrointestinal tract (Fig. 10). Probiotics might also be useful in treating and preventing allergic diseases and may provide an alternative to the pharmacological approach in patients who require lifetime treatment and/or suffer from serious side effects or drug resistance development [215,216].

At the cellular level, probiotic micro-organisms have several possible modes of action. They may be able to directly inhibit or kill pathogenic bacteria, or inhibit the attachment of pathogens to the wall of the gastrointestinal tract. This could reduce the virulence of the pathogens. In addition, probiotics have also been implicated in direct interaction with the immune system and immunomodulation by probiotics is presumed to be one of the main mechanisms of probiotic action observed in inflammatory diseases of the gut and in allergic diseases [217,218]. *L. plantarum*, *L. acidophilus* and *L. paracasei* are among the most predominant *Lactobacillus* species in the gut [219,220] and several strains within these species are marketed as probiotics. *Lactobacillus* strains are generally strong inducers of the pro-inflammatory cytokines TNF- α [221-223] and IL-12 [222,224-226], although several studies observed that the bacteria give a low or non-significant increase in IL-12 production [227,228]. Stimulation of IFN- γ production [225,226] or stimulation of the regulatory cytokine IL-10 could downregulate Th2 cytokine production which could be beneficial in allergic patients. In addition, *Lactobacilli* can upregulate the induction of Treg cells, triggering the release of regulatory cytokines (IL-10, TGF- β) and controlling the delicate balance between Th1 and Th2 immunity as well as tolerance [229-232].

In brief, probiotics may have a favourable role in immune disorders like allergy; however, further investigation on the possible mechanism and on possible effects are still required. In addition, it has been suggested that not all *Lactobacillus* strains exert a similar effect, therefore *in vivo* studies are often preceded by *in vitro* studies. In these *in vitro* studies several strains can be tested followed by a selection of the most promising strains to test in an *in vivo* setting. However, the correlation between *in vitro* and *in vivo* findings have rarely been studied [233], most probably because of a variety of parameters that interfere in the systematic comparison of strains such as the bacterial preparations used (viability, growth phase, dose and timing of administration) [234], the method of hPBMC preparation, and variable responsiveness of the donors. Even though probiotics might have a beneficial effect in several diseases, including allergy, more well-designed studies are needed to confirm these observations.

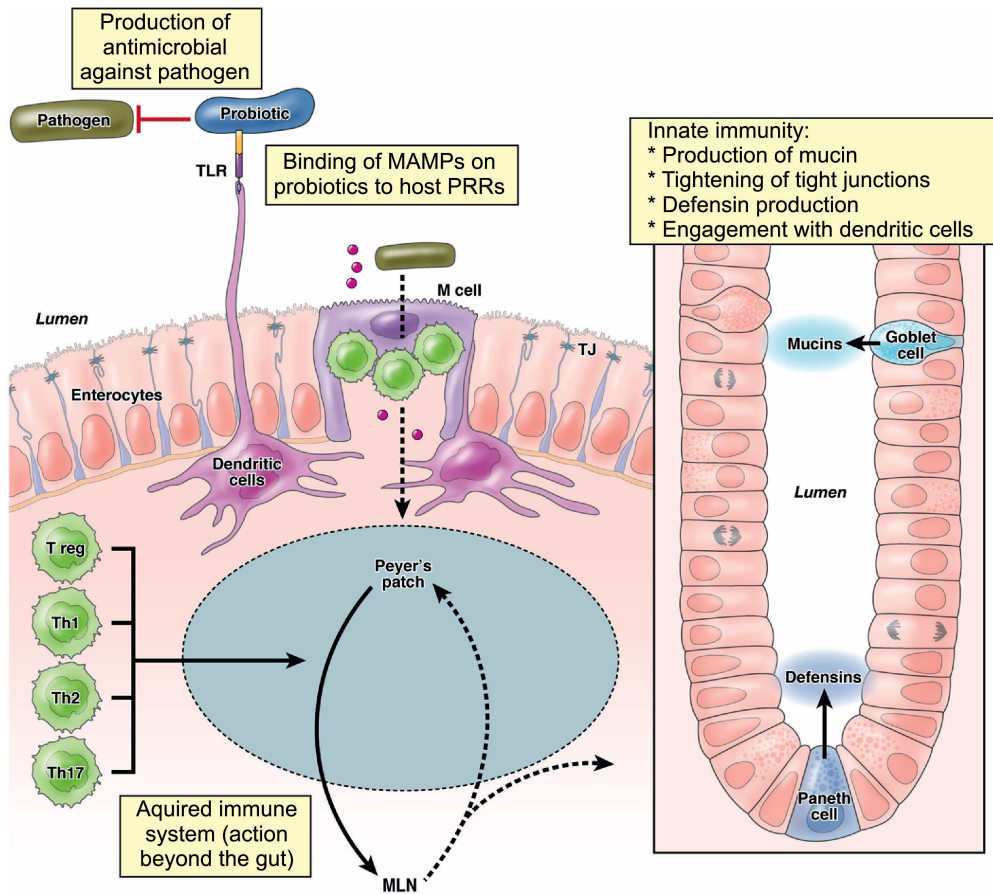


Fig. 10. Mechanisms of probiotic action: within the lumen, at the mucosal surface, and by stimulation of innate and acquired immunity. The predominant mechanism varies among different probiotic strains and is dependent on the clinical indication (Adapted from [235]).

Research aim and thesis outline

This thesis consists of two parts both centred around the theme of allergies. The first part of this thesis (chapter 2-6) studies the effect of processing on the allergenicity of purified allergens from peanut and hazelnut and contains a diagnostic paper on CRD in peanut/soy allergic patients. In the second part of this thesis (chapter 7-9) immunomodulating effects of *Lactobacillus* strains were studied, with as final aim to test whether consumption of selected strains could be beneficial for allergic patients. Fig. 11 shows an overview of the structure of this thesis.

Food allergens and processing

Changes in protein structure by processing methods might enhance or decrease IgE binding, but the changes by processing might also enhance or hinder the digestibility of a particular protein. Several studies have shown altered rate and pattern of proteolysis by processing methods such as heating and high pressure treatment [236-238]. An explanation could, for example, lie in the fact that thermal treatment causes unfolding of a protein leading to an increased accessibility of the specific peptide bonds to digestive enzymes like trypsin and chymotrypsin, which increase the rate of hydrolysis. Against this, prolonged heating time causing aggregation of the protein could cause a lower accessibility of the specific bonds and thereby decrease the rate of hydrolysis. This altered digestibility could modify the form in which allergens are taken up across the gut mucosal barrier and presented to the immune system with regards to both sensitisation and elicitation.

The effect of wet-heating and glycation on protein structure, IgE-binding properties and biological activity (i.e. mediator releasing capacities and T-cell proliferation and cytokine induction capacity) of peanut allergens is studied in **chapter 2** (Ara h 2/6) and **chapter 3** (Ara h 1). In addition, in chapter 2 and chapter 3 respectively, Ara h 1 and Ara h 2 and 6 from roasted peanuts were isolated, tested and results were compared to the effect of processing on the purified allergens. **Chapter 4** is also focussed on Ara h 1 and Ara h 2/6; however, now the effect of roasting (thus dry-heating) is studied on both IgE binding and degranulation capacities. In **chapter 5** we focus on the 7S globulin from hazelnut, Cor a 11, and study the effect of heating at three different temperatures in the presence or absence of glucose. The extent of Maillard reaction, IgG and IgE binding capacity and degranulation capacity were assessed using in the latter test both sera high in Cor a 11 specific IgE and sera from peanut allergic patients only containing cross-reactive IgE. **Chapter 6** consists of a short diagnostic paper in which we determined IgE profiles to peanut and soy allergens from adult peanut and/or soy allergic patients to investigate whether component-resolved diagnosis could help to predict clinical relevance of soy sensitization.

Immunomodulatory effects of lactic acid bacteria

As *in vitro* studies show that probiotics may be beneficial in allergic subjects, suggest it to be interesting to test the preselected strains with immune cells from allergic individuals, and to examine allergen-specific stimulation in the presence or absence of different bacterial strains. This will reveal whether the strains suppress the Th2 cytokine profile by stimulation of the Th1 response or by induction of Treg cells. This approach can help in alleviating the symptoms observed when having an allergy. As a follow up, a human trial testing the immunomodulatory effects of consumption of specific selected stains should be performed. For our studies we have chosen birch- and grass pollen allergic patients as our research population as these are common seasonal allergies. The prevalence of pollen allergy is estimated to be up to 40%, depending on tested age group, country etc. [239], with the major allergen from birch pollen (*Betula pendula* also *Betula verrucosa*), Bet v 1, as the most allergenic tree pollen in Northern, Central and Eastern Europe [240]. Therefore, a possible benefit of the use of probiotic strains could be of use for a large part of the population. In addition, birch-pollen allergic patients are easier to include compared to food allergic patients as the number of pollen allergic patients is much higher. However, assuming mechanistic similarities between respiratory and food aller-

gies, probiotics that demonstrate to be beneficial in pollen allergic patients, might be considered to explore for possible benefits to food allergic patients.

We started our research described in **chapter 7** by using hPBMC from healthy donors to assess the immunomodulatory effects of *Lactobacillus* species on the cytokine profiles and proliferative response of these hPBMC. From all tested strains, six were selected and now tested for potential immunomodulatory effects on hPBMC isolated from pollen-allergic patients in **chapter 8**. **Chapter 9** is the follow up of our *in vitro* studies in which oral treatment with five *Lactobacillus* strains are tested in a double-blind, placebo-controlled parallel study for their immunomodulatory properties in birch-pollen-allergic subjects.

Chapter 10 concludes this thesis with a general discussion on the results and conclusions from the previous chapters. It discusses the future perspectives for both allergy research with respect to the influence of processing as the use of probiotics in allergic patients.

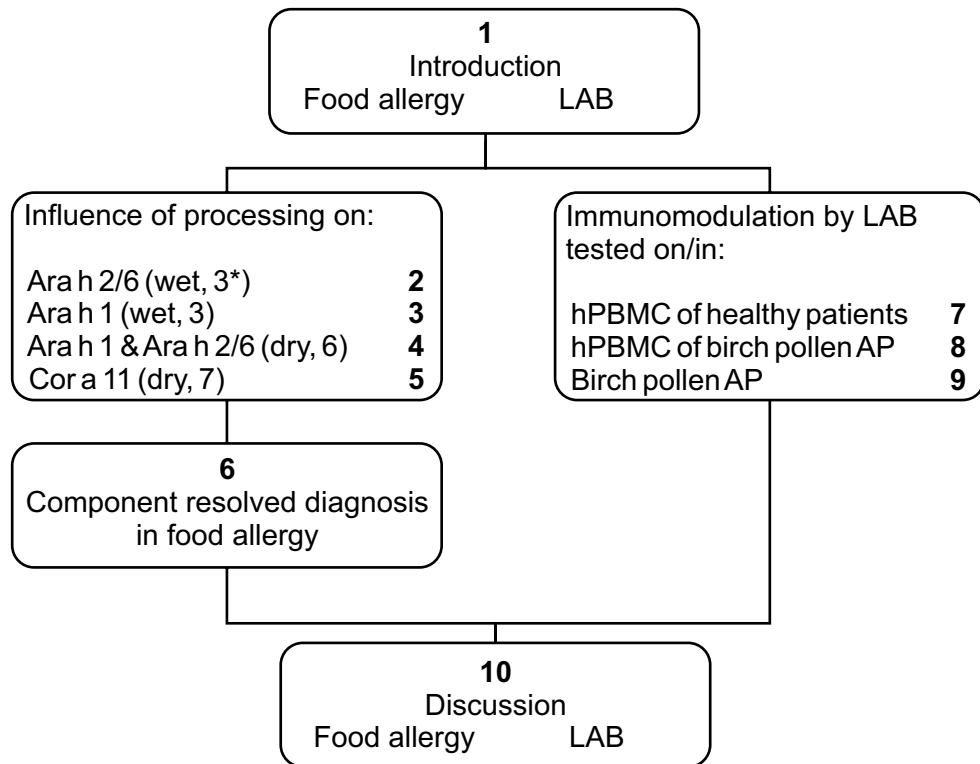


Fig. 11. Overview of the structure of the thesis *number of treatments tested; hPBMC: human peripheral blood mononuclear cells; LAB: lactic acid bacteria; AP: allergic person; wet/dry: wet or respectively dry-processing methods.

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Chapter 2

Effect of heating and glycation on the allergenicity of 2S albumins (Ara h 2/6) from peanut

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Submitted paper

Abstract

Background: Peanut allergy is one of the most common and severe food allergies, and processing is known to influence the allergenicity of peanut proteins. We aimed to establish the effect of heating and glycation on the IgE-binding properties and biological activity of 2S albumins (Ara h 2/6) from peanut.

Methodology/Principal Findings: Native Ara h 2/6 was purified from raw peanuts and heated in solution (15 min, 110°C) in the presence or absence of glucose. Ara h 2 and 6 were also purified from roasted peanut. Using PBMC and sera from peanut-allergic patients, the cellular proliferative potency and IgE reactivity (reverse EAST inhibition) and functionality (basophil degranulation capacity) of allergens were assessed. Heating Ara h 2/6 at 110°C resulted in extensive denaturation, hydrolysis and aggregation of the protein, whilst Ara h 2 and 6 isolated from roasted peanut retained its native conformation. Allergen stimulation of PBMC induced proliferation and Th2 cytokine secretion which was unaffected by thermal processing. Conversely, IgE reactivity and functionality of Ara h 2/6 was decreased by heating. Whilst heating-glycation further reduced the IgE binding capacity of the proteins, it moderated their loss of histamine releasing capacity. Ara h 2 and 6 purified from roasted peanut demonstrated the same IgE reactivity as unheated, native Ara h 2/6.

Conclusions/Significance: Although no effect of processing on T-cell reactivity was observed, heat induced denaturation reduced the IgE reactivity and functionality of Ara h 2/6. Conversely, Ara h 2 and 6 purified from roasted peanut retained the structure and IgE reactivity/functionality of the native protein which may explain the allergenic potency of this protein. Through detailed molecular study and allergenicity assessment approaches, this work then gives new insights into the effect of thermal processing on structure/allergenicity of peanut proteins.

Introduction

Peanut allergy is relatively common in the USA and certain European countries with the prevalence of sensitization being estimated as 2% and clinical peanut allergy as 1.2% of 3-4 years old children in the UK [1]. Whilst the incidence appears to be stabilising in the UK [1], it is still rising in the USA [2]. The peanut 2S albumins Ara h 2 and Ara h 6 together with a third low abundance 2S albumin, Ara h 7 have been identified as major peanut allergens [3-5]. **Ara h 2 and 6** comprise several isoforms of Mr 17 kDa and 15 kDa, respectively [6-8]. Produced as a single chain precursor they are proteolytically processed in peanut seeds into two subunits linked by intramolecular disulphide bonds [6,9]. Ara h 2, 6 and 7 are all members of the prolamin superfamily and share a characteristic cysteine skeleton with at least 8 conserved cysteine residues [9] and a three-dimensional structure comprising 5 α -helices arranged in a right-handed super helix. It appears this scaffold is stable to thermal processing and proteolysis [7,10,11].

Thermal processing of proteins can lead to alterations in their structure that can result in changes in their immunoreactivity/allergenicity. Typically, loss of tertiary structure is followed by reversible unfolding, while loss of secondary structure (70-80°C) leads to the formation of new intra/intermolecular interactions, rearrangements of disulfide bonds (80-90°C), and for-

mation of aggregates (90-100°C) [12]. Heating in the presence of sugars found in the foods also leads to modification by the Maillard reaction (non-enzymatic browning). Free primary amino groups are attacked by carbonyl compounds during the Maillard reaction, leading to the formation of stable advanced glycation end products (AGE). Several studies have been performed to assess the IgE-binding capacity of purified allergens modified *in vitro* by heating and/or by Maillard reactions. In some cases, glycation of allergens enhanced their IgE binding capacity [13] or their T-cell immunogenicity [14,15] whereas in other studies, glycation had no effect or caused even decreased IgE-binding capacity [16,17]. **Heating for 90 min at 100°C of recombinant refolded Ara h 2** led to a slight increase in its IgE binding capacity, which was further enhanced in the presence of glucose, maltose or ribose [18]. Heating native Ara h 2 for several days at 55°C in the presence of different sugars increased its IgE binding capacity compared to protein heated without sugar, which was related to the formation of AGE products [19]. Ara h 2 extracted from heat-processed peanut, such as roasting (140°C) was also found to enhance its IgE-binding capacity [20].

Although IgE binding capacities of modified allergens have been studied, sometimes with conflicting results, few data are available on the impact of heating on the protein structure and on the resultant biological activity of modified allergens compared to unmodified ones. In order to give new insights into the effect of thermal processing on structure/allergenicity of peanut proteins, we then purified and produced well-characterized native, heated and glycated Ara h 2/6, as well as corresponding protein from roasted peanut. Using a large panel of sera and peripheral blood mononuclear cells (PBMC) from well-characterized peanut-allergic patients recruited in different European countries, we then investigated the effect of thermal modifications on IgE reactivity of Ara h 2/6, but also on its biological activity, i.e. basophil activation, T-cell induced proliferation and cytokine production capacities.

Materials and Methods

Ethics statement

A written informed consent was obtained before the sample collection (serum or serum and PBMC) and the performed experiments were approved by the corresponding local ethical committees (Kantonale Ethikkommission Zürich, Medical ethical committee of the Amsterdam Medical Centre, Ethics Committee of Medical University of Vienna, Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen).

Patient characteristics

Thirty-four peanut-allergic (PA) patients (17 males and 17 females, mean age: 23 years) were recruited in Zurich, Amsterdam, Vienna and Arnhem, or were provided by the EuroPrevall Serum Bank (EPSB) (Table 1). Peanut allergy was established using clinical history, physical examination, peanut specific IgE (ImmunoCAP and/or RAST (Phadia AB)) and/or objective clinical manifestations observed after peanut ingestion. Grading of food-induced anaphylaxis was according to severity of clinical symptoms [35,36]. Twelve PA subjects and 12 non-allergic (NA) controls, all recruited from the Allergology Practice Arnhem (APA, the Netherlands), donated cells for PBMC cultures.

Peanut 2S albumins (Ara h 2/6) preparations

Native 2S albumins containing both Ara h 2 and Ara h 6 (N-Ara h 2/6) were purified from unroasted redskin type peanut as described previously [21] and stored at -20°C prior to use. This purification involves ammonium sulphate fractionation and gel-filtration chromatography, avoiding the use of denaturing conditions such as reverse phase HPLC. Allergen identity was confirmed by in gel-trypsin digestion and MALDI mass spectrometry. Ara h 2 and Ara h 6 were purified from commercial roasted peanut as previously described [20] which involves anion exchange chromatography in 4 M urea followed by reverse phase HPLC, and then mixed (62% Ara h 2 and 38% Ara h 6) in proportions representative of proportions found in peanut [37] to provide a preparation further referred to as R-Ara h 2/6.

Proteins were standardized according to BCA kit from Pierce, following provider recommendations.

Heating and glycation treatments of peanut 2S albumins (Ara h 2/6)

Native Ara h 2/6 (N-Ara h 2/6, 4 mg/ml) in 32.5 mM phosphate buffer containing 100 mM NaCl, was heated for 15 min to 110°C alone (H-Ara h 2/6) or in the presence of 100 mM glucose (G-Ara h 2/6). Heating in the presence of glucose was undertaken to glycate the protein based on a previously used protocol [38]. Protein solutions were allowed to cool to room temperature prior to analysis.

Biochemical characterization of native and modified Ara h 2/6

Changes in Ara h 2/6 secondary structure were assessed using far-UV (190-260 nm) circular dichroism (CD) spectroscopy using either a J-710 or a JASCO-810 spectropolarimeter (Jasco Ltd., Japan) as previously described [7,21]. **All data were calculated in terms of molar ellipticity.** Aggregation state was monitored using size exclusion chromatography on a Superdex-75 column attached to an Äkta Basic FPLC (Amersham Biosciences, Little Chalfont, UK) [21], and calibrated with a set of gel filtration molecular weight standards (BioRad, Hertfordshire, UK).

Isolation, culture and stimulation of human peripheral blood mononuclear cells

PBMC were isolated and cultured as previously described [39]. Immunological phenotyping of freshly isolated PBMC was performed on a FACS Canto II (BD Pharmingen, San Diego, USA), using monoclonal antibodies and the procedure from BD Pharmingen (San Diego, USA). Stimuli were incubated for at least 30 min with polymyxin B (50 µg/ml, Sigma, Zwijndrecht, the Netherlands) to remove potential endotoxin activity prior to addition of the stimuli to the PBMC. PBMC were stimulated with αCD3/αCD28 (150 ng/ml αCD3, 100 ng/ml αCD28) or N-, H- or G-Ara h 2/6 (20 µg/ml) and supernatants harvested after 4 and 7 days of culture and stored at -80°C. The 20 µg/ml dose was determined as optimal in preliminary studies (data not shown). Early apoptosis and late apoptosis/necrosis was assessed on freshly prepared and 7 day cultured cells using double staining with APC-Annexin V and propidium iodide (PI) [40]. PBMC proliferation was studied on day 4 and day 7 by analysis of intracellular expression of the nuclear Ki-67 antigen (BD Pharmingen, San Diego, USA) [40] of PBMC previously stained with anti-CD4/CD25 antibody. Ki-67 expression by CD4⁺ and CD25⁺ cells was then determined by flow cytometry.

Cytokine analysis

Cytokine production by PBMC was analysed in supernatants of cells cultured for 4 and 7 days. The production of IL-5, IL-10, IL-13, IFN- γ and IL-17 by PBMC cultures was determined in harvested supernatants using Cytometric Bead Array (CBA, BD Biosciences, San Diego, CA) on a FACSCanto II cytometer and the procedure was performed according to the manufacturer's protocol. The detection limit was 1.1 pg/ml for IL-5, 0.13 pg/ml for IL-10, 0.6 pg/ml for IL-13, 1.8 pg/ml for IFN- γ and 0.3 pg/ml for IL-17.

IgE immunoreactivity

Concentrations of IgE specific to whole protein extract from raw peanut and to N-Ara h 2/6 were determined using enzyme allergosorbent tests (EAST) as previously described [3,20].

Reverse EAST inhibition was performed as described elsewhere [41] using anti-human IgE (mouse monoclonal, clone LE27) as a capture antibody, biotinylated N-Ara h 2/6 (200 ng/ml) as tracer and N-, H-, G- or R-Ara h 2/6 as inhibitors. N-Ara h 2/6 was labelled with biotin using EZ-link sulfo-NHS-LC biotin (Pierce, Rockford, IL). Assays were developed using streptavidin labelled with acetylcholine esterase (AChE) and Ellman's reagent. The absorbance of each well was measured at 414 nm and results expressed as B/B_0 , where B_0 and B represent the amount of native Ara h 2/6 tracer bound to captured IgE in the absence or presence of a known concentration of inhibitor, respectively.

Effector cells activation by natural and modified allergens

Basophil histamine release (BHR) tests were performed as described in [42] using stripped basophils from fresh buffy coats (Blood Bank, National University Hospital of Copenhagen, Denmark) and reagents from RefLab (Copenhagen, Denmark). Twenty-three sera out of the 34 available were used individually in the BHR test, corresponding to all sera from Zurich, Amsterdam, Vienna and EPSB, and sera 54A to 58A from Wageningen.

RBL-2H3 cells expressing the α -chain of the human Fc ϵ R1 receptor [43] were kindly provided by Drs. Vieths and Vogel (Paul-Ehrlich-Institut, Langen, Germany). RBL cells were cultured in MEM medium supplemented with 5% fetal calf serum and 1% glutamine (all from Gibco-BRL, Paisley, UK) at 37°C in a humidified atmosphere with 5% CO₂. Cells in stationary growth phase were harvested and plated in 96-well plates at 1.5×10^5 cell/well. Six sera from PA patients (#65, #66, #67, #70, #55A and #56A) at pre-determined convenient dilutions and two sera from non-allergic patients were added and incubated overnight to passively sensitize the cells. After washing, the cells were stimulated for 1 h with the allergens diluted in Tyrode's buffer containing 50% deuterium oxide [43]. The antigen-specific release was quantified by measuring β -hexosaminidase activity and expressed as percentage of the total β -hexosaminidase content that was obtained by lysing the cells with Triton-X100 (Sigma-Aldrich, Zwijndrecht, the Netherlands). The release data were fitted to four-parameter-logistic curves by non-linear regression using the SigmaPlot 10.0 software package, and EC50 values were calculated accordingly.

Statistical analysis

Statistical analysis was performed by using SPSS (v18.0, SPSS Inc., Chicago, USA) or GraphPad Prism v4.00 for Windows (GraphPad Software, San Diego, CA). Means and medians were analysed using Wilcoxon signed rank test for specific IgE levels and PBMC cytokine production and

the Mann-Whitney U test for comparing PBMC cytokine production between PA and the control patient groups. Differences were interpreted as significant when $P < 0.05$.

Results

Effect of thermal processing on Ara h 2/6 structure

Native Ara h 2/6 (N-Ara h 2/6) gave a far-UV CD spectrum typical of an α -helix rich protein (Figs 1A and 1B) consistent with previous studies [21] and was monomeric with a Mr of ~ 16 kDa (Fig. 1C) consistent with a mixed preparation of Ara h 2/6 [7].

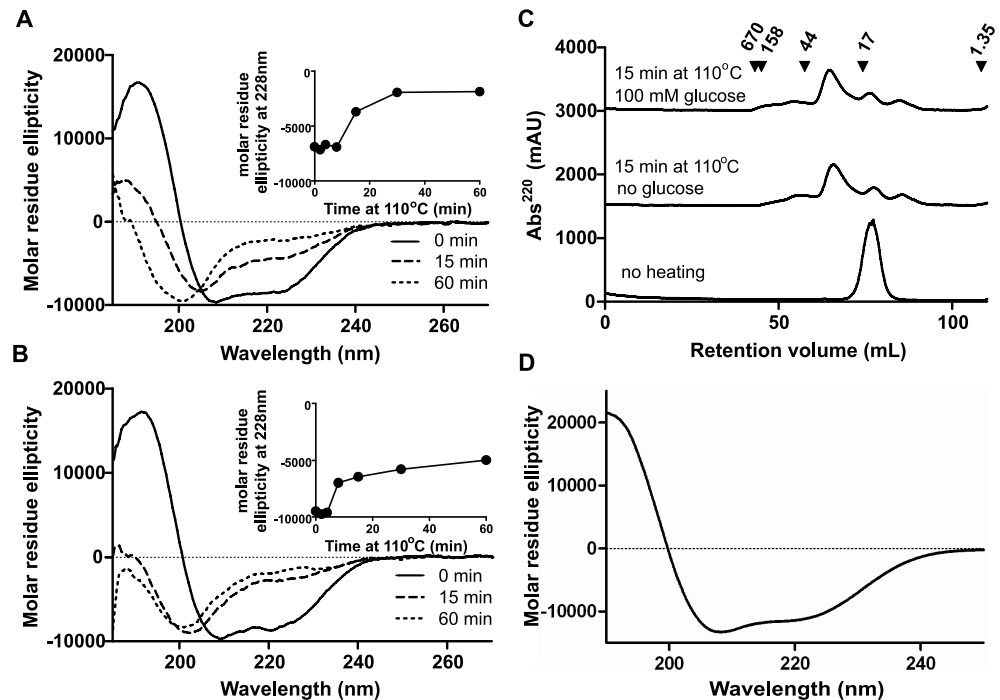


Fig. 1. Effect of heating on secondary structure and oligomeric state of Ara h 2/6. Far-UV CD spectra of Ara h 2/6 heated alone (**A**, H-Ara h 2/6) or in the presence of 100 mM glucose (**B**, G-Ara h 2/6) before heating (N-Ara h 2/6, —) and for heated-cooled protein after heating to 110°C for 15 min (---) and 60 min (----). Inset graphs show change in molar residue ellipticity at 228 nm with heating time. Size exclusion chromatography profiles (**C**) are shown of the Ara h 2/6 before and after heating for 15 min at 110°C in the presence or absence of glucose. Retention volumes of molecular weight standards (size indicated in kDa) are shown by arrow heads. **D.** Far-UV CD spectra of Ara h 6 purified from roasted peanut.

After heating at 110°C for 5-10 min in the absence [H-Ara h 2/6, Fig. 1A] or presence [G-Ara h 2/6, Fig. 1B] of glucose secondary structure shifted toward an unordered state which dominated after 15 min heating, with a loss of both the maximum at 198 nm and the minima at

209 and 222 nm. Heating in the presence or absence of glucose also affected the aggregation state of the protein, with only ~20% of the protein remaining in its monomeric state (Fig. 1C). A broad peak eluting with a Mr ~28-30 kDa of aggregated protein, together with a smear of material of Mr 44-150 kDa corresponding to larger oligomers was observed. In addition, thermal treatment resulted in hydrolysis of the Ara h 2/6 to yield fragments of smaller size than the parent protein. Far-UV CD spectra obtained for Ara h 2 (data not shown) and Ara h 6 (Fig. 1D) purified from roasted peanut were comparable and demonstrated the same α -helix rich protein structure as N-Ara h 2/6. Non-denaturing electrophoresis showed the proteins were mainly monomeric (data not shown).

In order to study the effect of heat-induced structural changes on allergenicity of 2S albumins, N-Ara h 2/6 was then further heated for 15 minutes at 110°C in the presence or absence of glucose.

Effect of thermal processing on PBMC induced proliferation and cytokine production capacity

No difference was observed between PBMC from PA or NA patients regarding differentiation markers, viability and cytokine secretion potency at the time of sampling and/or after polyclonal activation using α CD3/ α CD28 antibodies (data not shown). Stimulation with Ara h 2/6 did not induce significant proliferation in the NA controls (Fig. 2A) and only three of the 12 PA subjects showed detectable numbers of Ki-67+ proliferating cells ($10.5 \pm 4.8\%$) in the Ara h 2/6 stimulated cultures compared with medium alone (4.7 ± 2.7). The CD4+CD25+ subset was the largest cell population present in the corresponding proliferating PBMC. Thermal treatment of N-Ara h 2/6 had no effect on its capacity to induce PBMC proliferation in the three subjects (Fig. 2B).

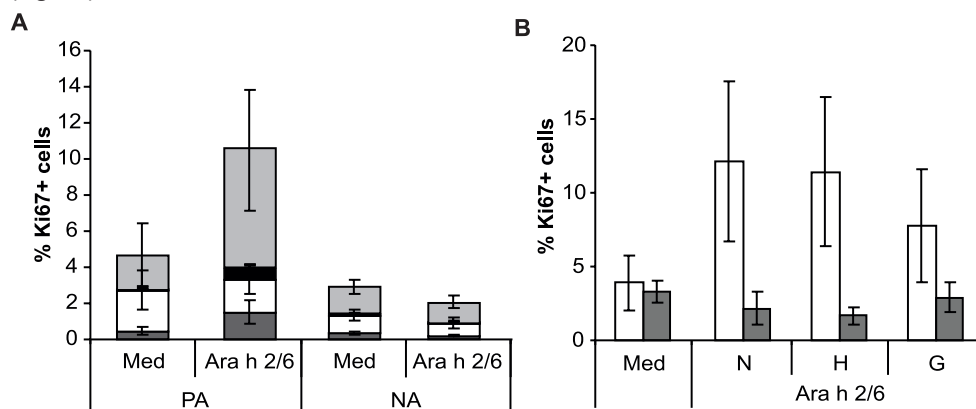


Fig. 2. Effect of thermal treatment on subpopulation of proliferating cells. **A.** Fresh PBMC were left unstimulated (Med) or stimulated with N-Ara h 2/6 for 7 days. After harvesting, the cells were incubated with anti-CD4/CD25 antibody and afterwards stained for an anti-Ki-67 PE antibody. Data are mean percentage of Ki-67 positive cells \pm SEM values for three (#65, #66 and #70) of the 12 PA subjects and seven NA controls. The percentage Ki-67+ cells was divided in the following subpopulations of cells: CD4-CD25- (dark grey bars), CD4+CD25- (white bars), CD4-CD25+ (black bars) and CD4+CD25+ (light grey bars). **B.** Fresh PBMC were left unstimulated (Med) or stimulated with N-, H- or G-Ara h 2/6 for 7 days. Data are mean percentage of Ki-67 positive cells \pm SEM values for three peanut-allergic patients (#65, #66 and #70) (white bars) and seven non-allergic controls (grey bars).

IL-5 and IL-13 production was significantly and equally enhanced in PBMC cultures from 5 out of 12 PA subjects following stimulation with N-, H- or G-Ara h 2/6 (Figs 3A and 3B), and not in PBMC cultures from NA subjects (Fig. 3 and data not shown). IL-4 correlated with IL-5 and IL-13 secretion although with lower levels (data not shown). Enhanced production of IL-10 and IFN- γ was observed in stimulated PBMC of PA subjects compared to NA subjects but was not significant for all stimuli tested (Figs 3C and 3D). IL-17 production was below 20 pg/ml for all tested conditions (data not shown).

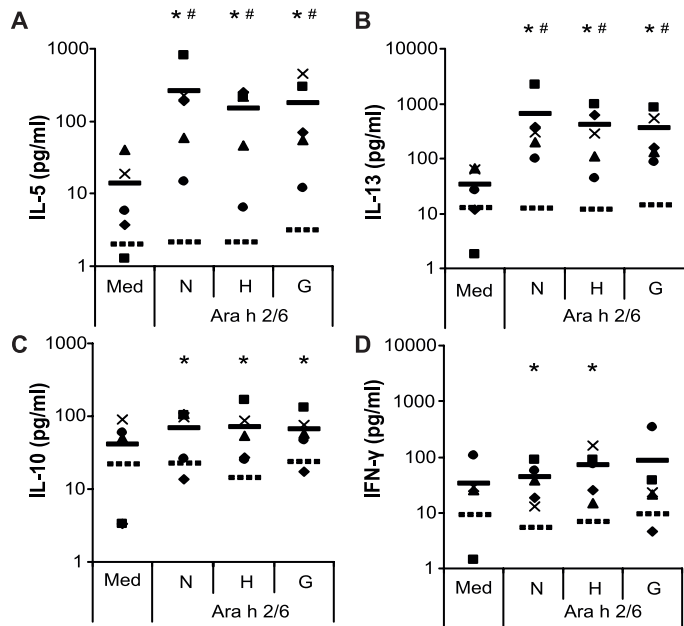


Fig. 3. Effect of thermal treatment on cytokine induction capacity. Fresh PBMC were left unstimulated (Med) or stimulated with N-, H- or G-Ara h 2/6 and production of IL-5 (A), IL13 (B), IL-10 (C) and IFN- γ (D) was measured after 7 days of culture. Symbols represent peanut-allergic patients #65 (■), #66 (◆), #70 (▲), #67 (●) and #73 (x). Horizontal bars represent the mean of the five PA responder subjects (solid) and the mean of 7 NA controls (dotted). Asterisks indicate statistically significant differences between the PA and the NA group and # indicate statistically significant differences between the medium and allergen-stimulated cultures for the PA group (*, # $P < 0.05$). No differences between the medium and allergen-stimulated cultures were observed for the NA group.

Effect of thermal processing on the IgE binding capacity

Screening of the serum panel ($n = 34$, Table 1) showed variable levels of IgE specific to raw peanut extract and N-Ara h 2/6. Four sera with low specific IgE were excluded from subsequent analysis (sera #69, #76, #80 and #82). Effect of thermal treatment on the IgE binding capacity of Ara h 2/6 was then assessed using reverse inhibition. A typical inhibition curve for the reverse EAST assay obtained with serum #05-0209 is shown in Figure 4A and demonstrated that the thermal treatment of N-Ara h 2/6 at 110°C reduced its IgE binding capacity. Ara h 2/6 from roasted peanut (R-Ara h 2/6) had a similar albeit slightly reduced immunoreactivity to N-Ara h

2/6 from raw peanut. IC₅₀ values calculated for the entire serum panel (n=30) are consistent with this and show that extreme thermal processing did reduce the IgE binding capacity of Ara h 2/6 in a significant manner (Fig. 4B). The IC₅₀ values for all tested sera increased 1.2 to 44-fold (mean = 9.6, n = 30) for H-Ara h 2/6 compared to N-Ara h 2/6 (Fig. 4B). For 25 out the 30 tested patients, IC₅₀ values for G-Ara h 2/6 were equivalent or 3.4-fold higher than those of H-Ara h 2/6 (mean = 1.7). For the last 5 patients, IC₅₀ values were 1.2 to 1.7-fold lower for G-Ara h 2/6 compared to H-Ara h 2/6 (mean 1.4). These data show that for most patients glycation reduced the IgE reactivity of Ara h 2/6 still further compared with thermal treatment alone, and is consistent with the fact that IC₅₀ values for G-Ara h 2/6 were 1.3 to 73-fold higher than that of N-Ara h 2/6 (mean = 14.5, n = 30). Ara h 2/6 from roasted peanut (R-Ara h 2/6) had a similar albeit slightly reduced immunoreactivity compared to N-Ara h 2/6.

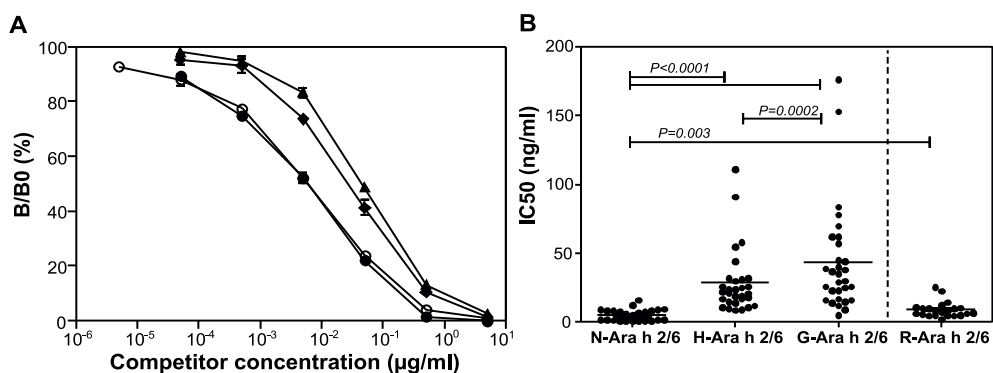


Fig. 4. Effect of thermal treatment on the IgE binding capacity of Ara h 2/6. **A.** IgE capture inhibition curves obtained for sera #05-0209 with N-Ara h 2/6 (●), H-Ara h 2/6 (◆) or G-Ara h 2/6 (▲). IgE binding capacities of native and heated processed Ara h 2/6 was assessed by competitive assays in the IgE capture format. Inhibition was performed into microtiter plates coated with anti-IgE, and previously incubated with allergic patient sera at convenient dilution. Competition was then conducted by adding increasing concentrations of competitors at the same time as biotinylated N-Ara h 2/6. Competition obtained with Ara h 2/6 purified from roasted peanut is shown as a clear circle. **B.** Analysis of IC₅₀ (ng/ml) values obtained using an IgE capture inhibition assay with native and heat processed Ara h 2/6 as competitors using 30 individual sera from peanut-allergic patients. Increase in IC₅₀ value corresponds to a decrease in IgE-binding capacity. R-Ara h 2/6: mix of Ara h 2 and Ara h 6 purified from roasted peanut. Bars indicate a significant difference between the 2 corresponding treatments ($P < 0.05$, non-parametric Wilcoxon signed rank test).

Table 1. Serological and clinical information for the 34 peanut allergic patients recruited in different European centres, within the EuroPrevall European project, used in EAST studies, basophil histamine release tests and/or PBMC proliferation tests.

Code	Gen-der (f/m)	Age	Peanut SPT (HEP Index numbers)	Grade peanut allergy	Peanut RAST class	Peanut CAP (IU/mL)	Anti-whole peanut (IU/mL)	anti-Ara h 2/6 IgE (IU/mL)	Other food allergies
2A	m	31	positive	3/4	5	51.7	43	31	
52A	m	41	nd	4	5	nd	166	62	
54A	f	11	2.2	1	6	>100	72	57	
55A	f	14	3.3	4	6	>100	363	137	
56A	m	16	1.5	2	6	>100	79	56	
58A	f	22	2.5	4	6	>100	171	110	Soy, Lupine, Pea
65	f	19	positive	3/4	6	>100	587	208	
66	m	18	positive	4	6	>100	290	71	
67	f	46	nd	3	5	54.4	95	35	Hazelnut, Kiwi, Cherries
68	f	20	positive	1	4	17.6	16	6	
69	m	21	positive	3/4	3	6.98	3.5	1.2	
70	f	21	positive	3/4	6	>100	224	76	Soy, Pea, Apple
73	f	22	positive	2/3	4	33.6	21	3.7	Hazelnut
76	m	23	positive	3	3	8.6	4.2	0.3	
77	m	25	positive	2	4	33.2	22	3.5	
78	f	24	positive	3	5	79.6	65	29	Hazelnut
80	m	27	positive	3	3	16	12	17	
82	f	18	positive	1/2	3	4.7	25	6.4	Kiwi, Cherries
1682	m	33	nd	4	6	>100	13	10	
1691	m	22	nd	3	5	nd	50	54	
2207	m	28	nd	4	4	nd	38	26	Wheat, Hazelnut, Egg, Almond
2209	f	27	nd	4	6	nd	75	51	Hazelnut, Egg, Wheat
2304	m	25	nd	3	6	nd	90	50	
2305	f	18	nd	4	nd	nd	52	36	
03-0043	f	20	1.54	3/4	6	>100	nd	852	Hazelnut, Walnut, Melon
03-0044	m	23	4.33	3	6	>100	nd	231	Egg, Fish, Shrimp, Hazelnut, Walnut, Sesame, Wheat, Sunflower
03-0079	f	16	2.22	3	6	>100	nd	88	Pistachio, Almond, Brazil nut
05-0128	f	25	1.92	3	6	>100	nd	358	Soy, Hazelnut, Apple, Peach
05-0165	f	18	0	3/4	6	>100	nd	429	Chickpea, Bean, Hazelnut, Lentil
05-0195	m	31	2.13	3	5	63.5	nd	64	Soy
05-0209	m	20	99.98	4	5	70.2	nd	81	Pea, Soy, Bean
06-0010	m	19	2.2	3/4	5	67.4	nd	67	Fish
08-0069	m	17	2.08	1	6	>100	nd	139	Kiwi
12-0048	f	21	5.8	3/4	5	54.6	nd	88	Shrimp

HEP: histamine equivalent prick test; nd: not determined. Where known, other allergies of the subjects are indicated. The origin of the sera is as follows: 2A: Zurich, 52A: Amsterdam, 54A-82: Arnhem, 1682-2305: Vienna; 03-0043 till 12-0048 EuroPrevall Serum Bank.

Grading is based on Brockow and Ring and on Sampson *et al.* [35,36]. Grade 1 = dermal symptoms; grade 2 = gastrointestinal problems like nausea and or cramping; grade 3 = any of the former grades plus vomiting/diarrhoea and respiratory tract problems like throat pruritus or tightness, grade 4 = any of the former grades and respiratory arrest plus cardiovascular problems like hypotension.

Effect of thermal processing on the mediator-releasing capacity

Subsequently an analysis of the effects of heating on the histamine releasing capacity of the Ara h 2/6 preparations was undertaken using human stripped basophils passively sensitized with 23 of the 34 peanut-allergic patients sera and compared with whole protein extract from raw or roasted peanut. No histamine release was induced by the different allergens when using a serum from patients not sensitized to peanut (data not shown), and the 23 sera from PA patients gave similar results. An example curve (serum #2305, Fig. 5A) shows significant histamine release for both extracts and purified allergens, the whole protein extract from raw and roasted peanut being comparable. As previously observed using RBL SX38 cells and Ara h 2 and Ara h 6 from roasted peanut [22], histamine release was induced at lower concentrations of these purified allergens when compared to peanut extracts. Heating reduced the biological potency of the Ara h 2/6, (Fig. 5A, $P < 0.05$ when considering 23 sera), but intriguingly histamine release was induced at a lower concentration of heated-glycated than heated Ara h 2/6. The maximum histamine release induced by the purified allergens was only affected when comparing N-Ara h 2/6 to H-Ara h 2/6 ($P < 0.05$ when considering the 23 sera).

Additionally, a mediator release assay using humanized Rat Basophilic Leukaemia cells was also performed using 6 individual sera. Figure 5B shows typical results of a representative serum (#70) and the inserted table represents the average protein concentrations needed to obtain 50% of the maximum mediator release induced by the native allergen (EC₅₀). A 150 and 130 times increased EC₅₀ value, corresponding to a decreased allergenic activity, was observed for H-Ara h 2/6 and G-Ara h 2/6, respectively, in comparison to N-Ara h 2/6. The EC₅₀ value of G-Ara h 2/6 was lower compared to the EC₅₀ value of H-Ara h 2/6; however, this was not tested to be significantly different.

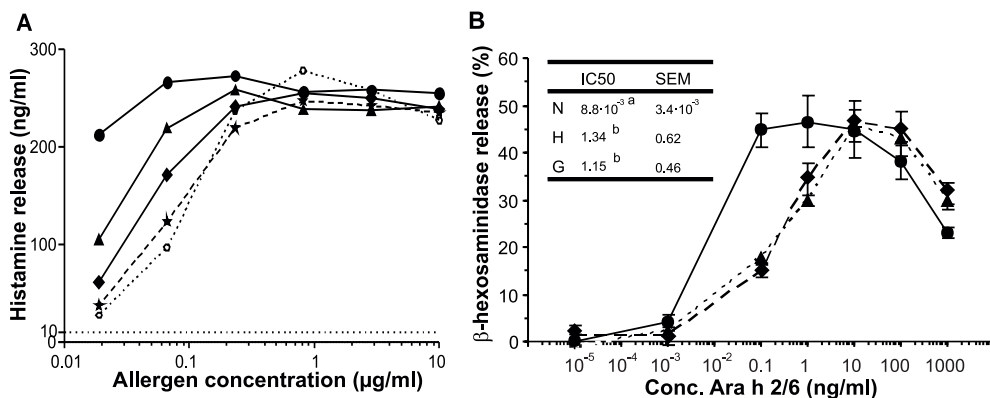


Fig. 5. Effect of thermal treatment on the activation of effector cells induced by Ara h 2/6. **A.** Human stripped basophils were passively sensitized with individual sera (#2305) and then incubated with increasing concentrations of N-Ara h 2/6 (●), H-Ara h 2/6 (◆), and G-Ara h 2/6 (▲), or whole peanut extract from raw (★) or roasted (○) peanut. Histamine release was assayed in corresponding supernatants. Twenty-three sera out of the 35 available corresponding to all sera from Zurich, Amsterdam, Vienna and EPSB, and sera 54A to 58A from Arnhem were used, giving similar results (not shown). No histamine release was induced by the different allergens when using a serum from patients not sensitized to peanut (data not shown). **B.** Humanized RBL-2H3 cells were passively sensitized with sera

from peanut-allergic patients (#70) and stimulated with increasing concentrations of native (N; —●—), heated (H; —◆—) or glycated (G; ---▲---) Ara h 2/6. Error bars represent the SD of triplicate values. No β -hexosaminidase release was induced by the different allergens when using sera from non-allergic patients (data not shown). The table presented within the figure represents the average protein concentrations (ng/ml, $n = 6$) to obtain 50% of the maximum allergen release of the native allergen (EC50). Means without a common letter differ ($P < 0.05$).

Discussion

Boiled peanuts are consumed in many countries, from China to the Southern states of the USA. Boiling is performed for 2 to 7 hours, depending on the peanut source, and boiled peanut can be further canned, then involving further extensive heating (45 minutes at 120°C under 10 pounds pressure per square inch). Study of heat-induced structural changes and its impact on allergenicity of peanut allergens after wet-thermal processing then appears of interest. Achieving model processing conditions to ensure that thermal modifications can be monitored by structural and immunological analysis is difficult since heating frequently renders much of the protein insoluble. This makes purification of proteins from cooked foods, such as roasted or boiled peanuts, cumbersome [23]. We then analyzed the effect of wet thermal processing on major peanut allergens, i.e. N-Ara h 2/6, previously purified from raw peanut. Like other members of the prolamin superfamily, the 2S albumin allergens of peanut appeared to be highly thermostable, properties which may contribute to their allergenic potency. Through our detailed molecular study we have shown that these proteins only begin to unfold following heating at temperatures over 100°C, conditions which equate to boiling for extended times (longer than 15 min). After this time, the protein adopts a random coil conformation and forms dimers and higher order oligomers, accompanied by hydrolysis of the peptide backbone. Such effects of thermal denaturation have been observed for other food proteins, including the lipid transfer protein from barley which shares the same protein scaffold as the 2S albumins [24]. Certainly the Ara h 2 and 6 purified from roasted peanuts had retained the structural characteristics of the native protein purified from raw peanuts, as indicated by far-UV CD spectra and non-denaturing electrophoresis. These data demonstrate that the proportion of the protein in roasted peanut that was still soluble had not been denatured by the roasting process. In addition, this proportion was shown to have a similar immunoreactivity as the unheated protein.

In order to assess the effect of heat-induced structural changes on allergenicity of Ara h 2/6, we first assessed the *in vitro* proliferative capacity of the native vs heated proteins using PBMC from 12 peanut allergic (PA) and 12 control human donors. Out of the 12 PA, only 5 demonstrated a significant cytokine production after *in vitro* stimulation. All of them had high levels of peanut specific IgE antibodies. This corroborates studies demonstrating a significant positive correlation between allergen-specific serum IgE levels and high numbers of IL-4-producing cells [25] or active proliferation of T cells and Th2 cytokine production [26]. However, it is worth noting that peanut-specific T-helper cells in PBMC of peanut allergic patients were found to be only 0.6% of the total CD4+ T cells [27]. This explains the quite low rate of responders in our *ex vivo* stimulation assays. Nevertheless, we could still conclude that heat treatment of Ara h 2/6 did not influence cytokine production by PBMC from either of the 5 PA. These results then demonstrated that the unfolding of the Ara h 2/6 by intense heating had no effect on the PBMC

stimulating activity of Ara h 2/6, as has been shown before for Bet v 1-related allergens [28,29].

The IgE-binding capacity and the elicitation potency of the native vs heat-processed allergens were then studied using a large panel of sera from peanut allergic patients ($n = 30$ and 23 , respectively). In fact, most of the studies aiming at analyzing the relationship between structure and allergenicity, and particularly the effect of processing on allergenicity of food proteins, generally use very few individual sera from allergic patients [18,23,30,31], even sometimes only one pool of such sera is available [11,19,32,33]. Our results show that heat-induced denaturation of Ara h 2/6 did result in a significant loss in IgE-binding capacity and ability of the protein to elicit histamine release. This indicates that thermal processing can reduce the allergenic activity of peanut proteins in terms of elicitation potential, and may explain why processes such as boiling of peanuts, can reduce their allergenic activity [32]. Our data also suggest that IgE responses in peanut-allergic individuals are directed towards the natively folded protein and/or that oligomerisation observed after heating with or without glucose led to epitope masking. Thus, it appears that the sensitizing agent driving B-cell responses in this population was native Ara h 2/6 which may have originated from either consumption of raw nuts, or more probably, represent the fraction of Ara h 2/6 that remains soluble and in its native state even after roasting.

We found that glycation in conjunction with thermal denaturation generally led to a further decrease of IgE binding capacity, rather than the increase found by others [18,34], although glycation did appear to preserve slightly more of the proteins mediator releasing capacity compared with heating alone. Some of these differences may relate to the wet-thermal processing procedures employed in this study. Further investigation using heat-denaturation of Ara h 2/6 under low moisture conditions will be required to explain these differences more fully.

In conclusion, we demonstrated that heating to temperatures able to denature Ara h 2/6 caused a significant decrease in the proteins allergenicity whereas T cell stimulation was not affected. It is evident that a soluble fraction of the Ara h 2/6 from roasted peanuts retains the conformation and allergenic activity of the native protein, explaining the allergenic potency of this protein.

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Chapter 3

Boiling peanut Ara h 1 results in formation of aggregates with reduced allergenicity

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Submitted paper

Abstract

Scope: It has been proposed that roasting *versus* boiling and Maillard modifications may modulate peanut allergenicity. We investigated how these factors affect the allergenic properties of a major peanut allergen, Ara h 1.

Methods and results: Ara h 1 was purified from either raw (N-Ara h 1) or roasted (R-Ara h 1) peanuts. Boiling (100°C 15 min; H-Ara h 1) resulted in a partial loss of Ara h 1 secondary structure and formation of rod-like branched aggregates with reduced IgE binding capacity and impaired ability to induce mediator release. Glycated Ara h 1 (G-Ara h 1) formed by boiling in the presence of glucose behaved similarly. However, H- and G-Ara h 1 retained the T-cell reactivity of N-Ara h 1. R-Ara h 1 retained no native secondary structure, comprised compact, globular aggregates, showed no evidence of glycation but retained the IgE binding capacity of the native protein.

Conclusion: Ara h 1 aggregates formed by boiling were morphologically distinct from those formed by roasting and had lower allergenic activity. Simple glycation did not affect Ara h 1 allergenicity. These data help to explain differences in the allergenicity of boiled and roasted peanuts and provides insights into how boiling may reduce elicitation capacity.

Introduction

Peanut allergy is a common, severe and generally persistent food allergy in the North America and several European countries [1]. It appears to be less prevalent in other parts of the world and it has been proposed that differences in cooking practices maybe responsible [2]. Different types of thermal processing (blanching, boiling, roasting or frying) will alter protein structure in different ways, potentially changing its immunoreactivity and allergenicity. Since foods are complex mixtures of many different constituents, notably sugars, cooking can also modify proteins chemically. One of the most important of these cooking-related changes is the Maillard reaction, or non-enzymatic browning, which leads to the formation of stable advanced glycation end products (AGE) through the reaction of reducing sugars with free amino groups on proteins. It has been proposed that this type of processing can also affect allergenicity of peanuts [2].

This has been particularly well studied for the peanut allergen Ara h 1, to which 55-95% of peanut-allergic patients are sensitized. Belonging to the 7S globulin protein family it possesses the β -barrel structural motif characteristic of the cupin superfamily [3-6]. When purified from raw peanut it is a Mr 210 kDa trimeric protein [7], composed of 63 kDa N-glycosylated subunits which can form multimers of up to Mr 600-700 kDa depending on extraction conditions [8]. Like other cupins Ara h 1 is thermostable, only undergoing irreversible denaturation and extensive aggregation after passing through the main endotherm transition occurring above 80°C [9]. However, more extreme thermal processing such as roasting at 140°C appears to enhance IgE-binding capacity of Ara h 1 [10]. In model systems heating native Ara h 1 for several days at 55°C in the presence of different sugars increased its IgE binding capacity, which was related to the formation of AGE products although the IgE binding capacity of the modified Ara h 1

and untreated allergen was not directly compared [11]. Such modifications may explain the enhanced IgE-binding capacity of Ara h 1 from roasted peanuts.

The problems of handling and investigating the structures of thermally aggregated proteins with reduced solubility has made it difficult to link effects of thermal processing on structure to effects on allergenic properties. In addition, few data are available comparing the functionality of modified and unmodified allergens. We have addressed this gap using structurally well-characterized thermally modified Ara h 1 preparations (including model processed Ara h 1 and the cognate protein from roasted peanuts). We investigated the effect of thermal treatment on both the allergen structure and its IgE binding capacity, potency to stimulate histamine release and ability to induce T-cell proliferation and cytokine production using sera and peripheral blood mononuclear cells (PBMC) from peanut allergic patients.

Materials and Methods

Ethics statement

A written informed consent was obtained before the sample collection (serum or serum and PBMC) and the performed experiments were approved by the corresponding local ethical committees (Kantonale Ethikkommission Zürich, Medical ethical committee of the Amsterdam Medical Centre, Ethics Committee of Medical University of Vienna, Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen).

Patient sera and peripheral blood mononuclear cells (PBMC)

Sera were obtained from a population of 35 peanut-allergic (PA) patients (18 males and 17 females, mean age: 23 years) recruited from clinical centers in Zurich, Amsterdam, Arnhem, and Vienna, or were provided by the EuroPrevall Serum Bank (EPSB) (Table S1). Twelve PA subjects (underlined in Table S1) and 12 non-allergic (NA) controls, all recruited from the Allergy Practice Arnhem (APA, the Netherlands), donated cells for PBMC cultures. Peanut allergy was established using clinical history, physical examination, peanut specific IgE (ImmunoCAP and/or RAST (Phadia AB; Table S1)) and objective clinical manifestations observed after peanut consumption (anaphylaxis in the history or positive food challenge).

Protein preparations

Whole Peanut Protein Extract (WPPE) was prepared from peeled raw and commercially roasted peanuts (Virginian variety) from which R-Ara h 1 was purified [10]. Native Ara h 1 (N-Ara h 1) was purified from a single batch of peeled redskin type raw peanuts (obtained from local suppliers, UK) as described by Marsh *et al.* [12]. N-Ara h 1 (4 mg/ml) in 32.5 mM phosphate buffer containing 100 mM NaCl, was heated to 100°C alone (heated Ara h 1, H-Ara h 1) or in the presence of 100 mM glucose (glycated Ara h 1, G-Ara h 1) for 15 min. Protein solutions were allowed to cool to room temperature prior to analysis. Changes in secondary structure were defined using circular dichroism and Fourier transform infrared spectroscopy [12]. Aggregation state was defined using atomic force microscopy (AFM) depositing protein (40 µg/mL diluted 50-fold from a 2 mg/mL stock solution with water) on to freshly prepared poly-L-lysine coated muscovite mica (Agar Scientific, UK). The sample was inserted into the liquid cell of the AFM

(East Coast Scientific, Cambridge, England) and imaged in contact mode at a set point force of around 200 pN under redistilled n-butanol (Sigma, Dorset, UK) for the aggregates and buffer for the unheated proteins using 100 μm long oxide-sharpened Nanoprobe levers (NP-S, Veeco Instruments Inc., California, USA) with a quoted force constant of $k = 0.38 \text{ Nm}^{-1}$. Complimentary analysis was undertaken using fixed angle light scattering carried out on a Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

IgE immunoreactivity to the native and modified Ara h 1 by direct and reverse EAST inhibition

IgE specific to whole protein extract from roasted or raw peanut and to N-Ara h 1 or R-Ara h 1 were determined using the EAST (enzyme allergosorbent test) as previously described on allergen-coated plates [3,10]. Reverse EAST inhibition was performed as described elsewhere [13] using anti-human IgE (mouse monoclonal, clone LE27) coated plates. Inhibitors consisted of increasing concentrations of R-, N-, H- or G-Ara h 1 using either N-Ara h 1 or R-Ara h 1 labeled with acetylcholinesterase (AChE) [14] as the tracer. Results were expressed as B/B_0 , where B_0 and B represent the amount of Ara h 1 tracer linked to immobilized IgE in the absence or presence of a known concentration of inhibitor, respectively.

Mediator release assay (MRA) using Rat Basophilic Leukemia (RBL) cells

RBL-2H3 cells expressing the α -chain of the human Fc ϵ RI receptor [15] were kindly provided by the Paul-Ehrlich-Institut (Langen, Germany) and MRA were performed as described previously (Vissers *et al.*, submitted) using human sera annotated with an asterisk in Table S1. Antigen-specific release induced by N-, H- and G-Ara h 1 was quantified by measuring β -hexosaminidase activity and expressed as percentage of the total β -hexosaminidase content that was obtained by lysing the cells with 1% Triton-X100 (Sigma-Aldrich, Zwijndrecht, the Netherlands).

Effect of thermal processing on PBMC induced proliferation and cytokine production capacity

PBMC were isolated from PA ($n = 12$) and NA ($n = 12$) donors and cultured according to previously described procedures ([16] and Vissers *et al.*, submitted). No differences in viability, proliferation or cytokine secretion could be evidenced between PA and NA after polyclonal activation with $\alpha\text{CD3}/\alpha\text{CD28}$ (suppl file 1). PBMC were stimulated with 20 $\mu\text{g}/\text{ml}$ of N-, H- and G-Ara h 1 or with medium alone, cultured for 7 days at 37°C and supernatants were stored as previously described (Vissers *et al.*, submitted). Harvested PBMC were characterized using flow cytometry using a FACSCanto II (BD Biosciences San Diego, CA, USA) as previously described (Vissers *et al.*, submitted) using labeled anti-CD4, anti-CD25 and anti-Ki-67 antibodies or the corresponding isotype controls, all from BD Pharmingen (San Diego, USA). Cytokine production was determined using Cytometric Bead Array (CBA, BD Biosciences, San Diego, CA), according to the manufacturer's protocol. The detection limits were 1.1 pg/ml for IL-5, 0.13 pg/ml for IL-10, 0.6 pg/ml for IL-13, 1.8 pg/ml for IFN- γ and 0.3 pg/ml for IL-17.

Statistical analysis

Means and medians were analyzed using nonparametric methods (Wilcoxon signed rank test or Mann-Witney U test) using SPSS Software (version 18.0, SPSS Inc., Chicago, USA) or GraphPad Prism (version 4.00 GraphPad Software, San Diego, CA) with a difference interpreted as significant when $P < 0.05$. MRA data were fitted to four-parameter-logistic curves by non-linear regression using the SigmaPlot 10.0 and EC50 values determined.

Results

Boiling (H-Ara h 1) resulted in aggregation and hydrolysis of Ara h 1 as indicated by the appearance of lower Mr polypeptides ranging from ~6-67 kDa and the generally smeared appearance of the gel track (Fig. 1A track 3) compared to the Mr ~ 67 kDa and ~ 33 kDa polypeptides of native (N-) Ara h 1 (Fig. 1A track 2) [12]. Addition of glucose during boiling (G-Ara h 1) resulted in the formation of high Mr polypeptides of masses > 200 kDa (Fig. 1A track 4). Ara h 1 isolated from roasted peanuts (R-Ara h 1) resembled the heated and glycosylated protein (Fig. 1A track 5) with much of the protein migrating as a poorly resolved smear at the top of the gel. Boiling, alone or in the presence of glucose caused a partial loss of secondary structure, as indicated by the loss of the positive maximum at 192-195 nm, and the positive molar ellipticity at 190 nm in the CD spectra of H- and G-Ara h 1 (Fig. 1B). However, the protein from roasted peanuts (R-Ara h 1) appeared to be highly denatured having only a slight positive maximum at 190 nm, and a negative minimum at 205 nm of reduced intensity (Fig. 1B).

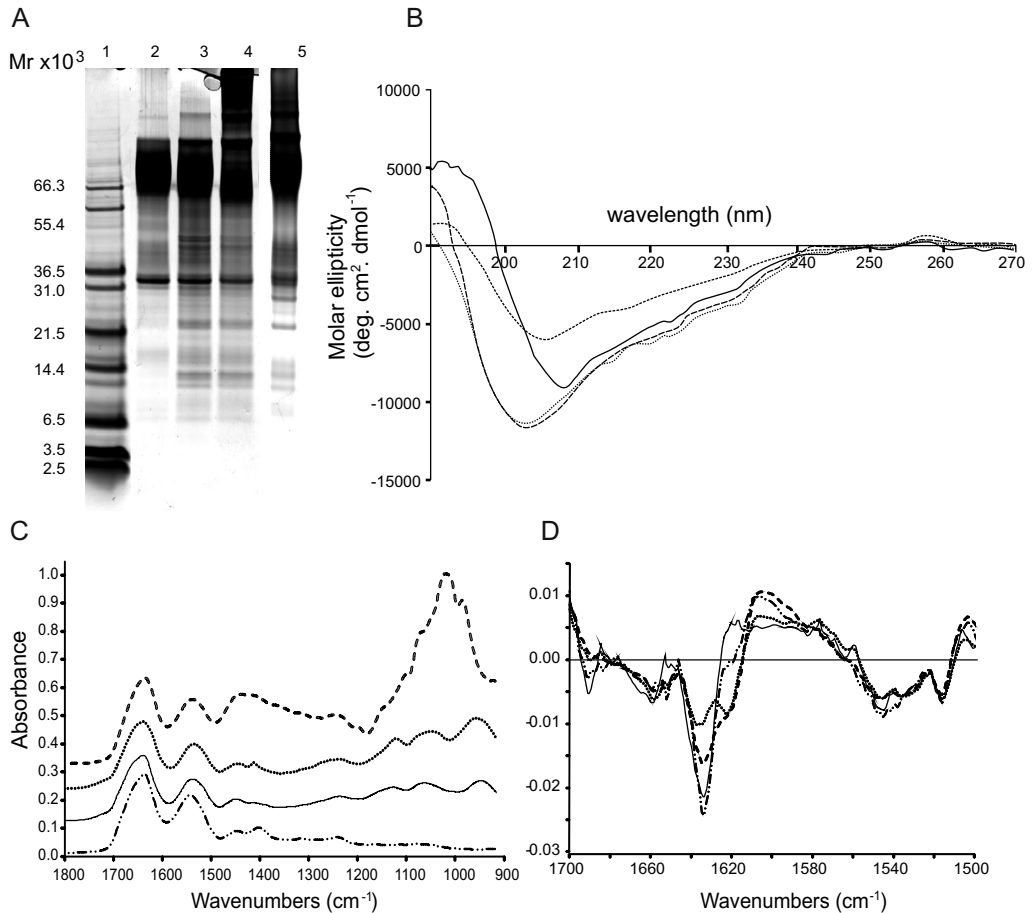


Fig. 1. (previous page) Physicochemical characterisation of heat-treated Ara h 1. **A.** SDS-PAGE analysis of Ara h 1 preparations. Track 1: Molecular weight markers; 2: native (N-) Ara h 1; 3: heated (H-) Ara h 1; 4: heated and glycosylated (G-) Ara h 1; 5: R-Ara h 1 from roasted peanuts. **B.** Circular dichroism (CD) spectra of native and heat treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (-----); G-Ara h 1 (- - -); R-Ara h 1 (- - -). **C.** Fourier transform infrared spectroscopy (FT-IR) spectra of native and heat treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (-----); G-Ara h 1 (- - -); R-Ara h 1 (- - -). **D.** Second derivatives of the amide band region of the Fourier transform infrared spectroscopy (FT-IR) spectra of native and heat treated Ara h 1. N-Ara h 1 (—); H-Ara h 1 (-----); G-Ara h 1 (- - -); R-Ara h 1 (- - -).

Complementary fourier transform infrared spectroscopic analysis (FT-IR) was undertaken to provide additional information on secondary structure content and to obtain an indication of glycation state of the different protein preparations (Fig. 1 C, D). The amide I band shape of N-Ara h 1 gave an amide I band maximum at 1634 cm^{-1} characteristic of the natively folded β -sheet rich protein, with a small shoulder at 1656 cm^{-1} corresponding to α -helix. After heating the 1634 cm^{-1} band was reduced in intensity and a new band appeared at 1621 cm^{-1} which is generally accepted as an indication of the formation of intermolecular β -sheet structures and protein aggregation [17]. Heating in the presence of glucose (G-Ara h 1) resulted in similar, but smaller, changes in secondary structure as well as glycation as indicated by the intense bands in the $1200\text{-}900\text{ cm}^{-1}$ region. Roasting, i.e. heating at high temperature in low moisture environment, had a completely different effect. The amide I band shape of R-Ara h 1 gave an even more pronounced amide I band maximum at 1634 cm^{-1} characteristic of the natively folded β -sheet rich protein, while the shoulder at 1656 cm^{-1} corresponding to α -helix was reduced. Only a small new band related to intermolecular β -sheet formation, appeared at 1621 cm^{-1} . Furthermore the R-Ara h 1 had only a very minor absorption in the $1200\text{-}900\text{ cm}^{-1}$ region, indicating a very limited level of glycation of the protein.

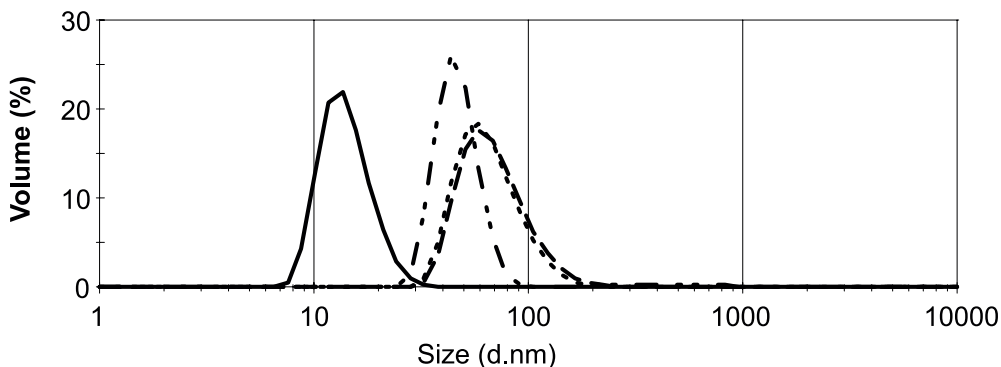


Fig. 2. Fixed angle light scattering analysis of heat-treated Ara h 1. Light scattering measurements of native and heat treated Ara h 1. N-Ara h 1 (—), H-Ara h 1 (— —), G-Ara h 1 (- - -) or R-Ara h 1 (- - -). Results are shown as size distribution by volume, percentage volume versus size in nm. Data represent the mean of three measurements per sample.

H-, G- and R-Ara h 1 were all highly aggregated with Mrs of $> 640,000$ by gel permeation chromatography and dimensions of in excess of 50 nm by fixed angle dynamic light scattering (Fig. 2). Characterization of boiled Ara h 1 aggregate topography using AFM showed they had

either a rod-like branched aggregate structure (H-Ara h 1, Fig. 3B) or simpler rod-like structures when glucose was added (G-Ara h 1, Fig. 3C) both with variable heights between 10-15 nm. The R-Ara h 1 aggregates had a very different morphology (Fig. 3D) and were either more globular or comprised slightly shorter rods with significantly lower heights of around 7-8 nm.

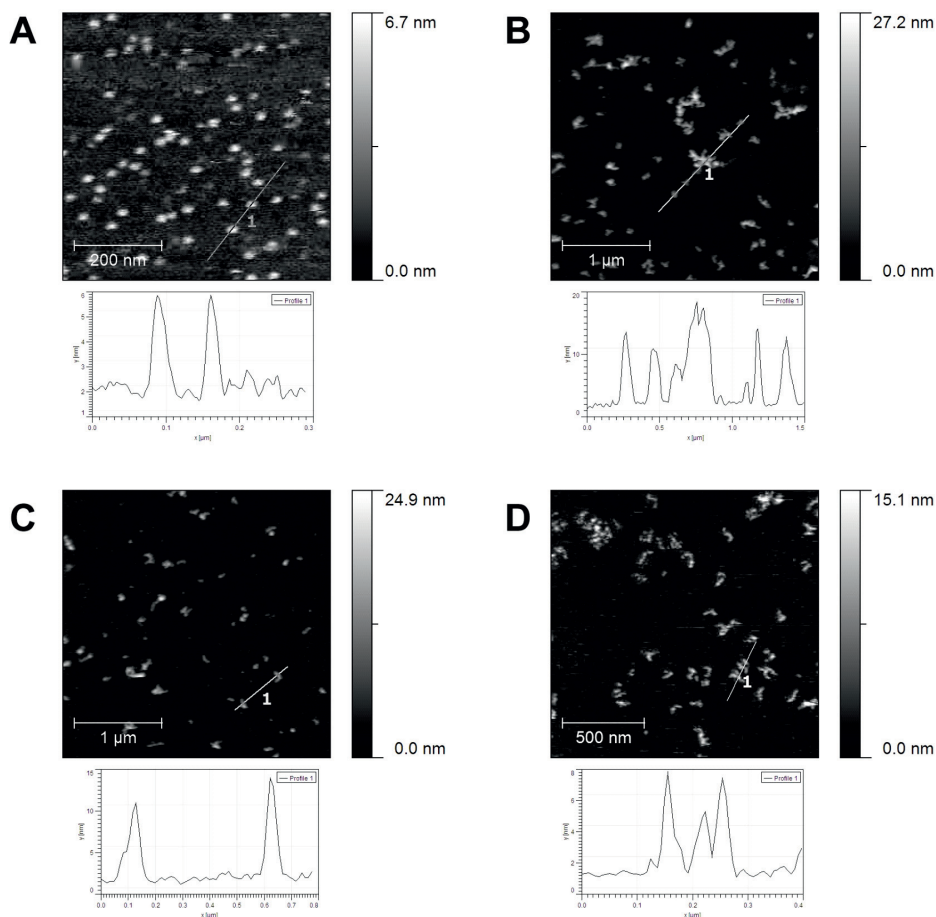


Fig. 3. Atomic force microscopy topographic images of heat-treated Ara h 1. **A.** N-Ara h 1; **B.** H-Ara h 1; **C.** G-Ara h 1; **D.** R-Ara h 1. Profiles beneath each image depict feature heights along marker line.

Boiled H- and G-Ara h 1 had greatly reduced IgE binding capacities for all patient sera analyzed using reverse EAST inhibition, although the pattern of reactivity was heterogeneous (Fig. 4). Typically boiling resulted in either a very slight (#2206, Fig. 4A) or more marked (#66, Fig. 4B) reduction in IgE binding capacity of Ara h 1. This was reflected in the increase in IC₅₀ values for H-Ara h 1 compared to N-Ara h 1 of 1.5 to 5800 fold (mean = 715) and 1.8 to ≥ 10000 fold (mean = 1236) for G-Ara h 1 (Fig. 4C). IC₅₀ values were higher for H- than for G-Ara h 1, for 5 out of 26 sera, showing for these patients that H-Ara h 1 was slightly, but significantly, more IgE

reactive, whilst the remaining 19 sera showed the opposite pattern of reactivity. Remarkably, the IgE immunoreactivity of the R-Ara h 1 was almost identical to that of N-Ara h 1, with mean IC₅₀ values of 31.6 and 37.3 ng/ml respectively, and showed less inter-individual variability than the N-Ara h 1 (Fig. 4C).

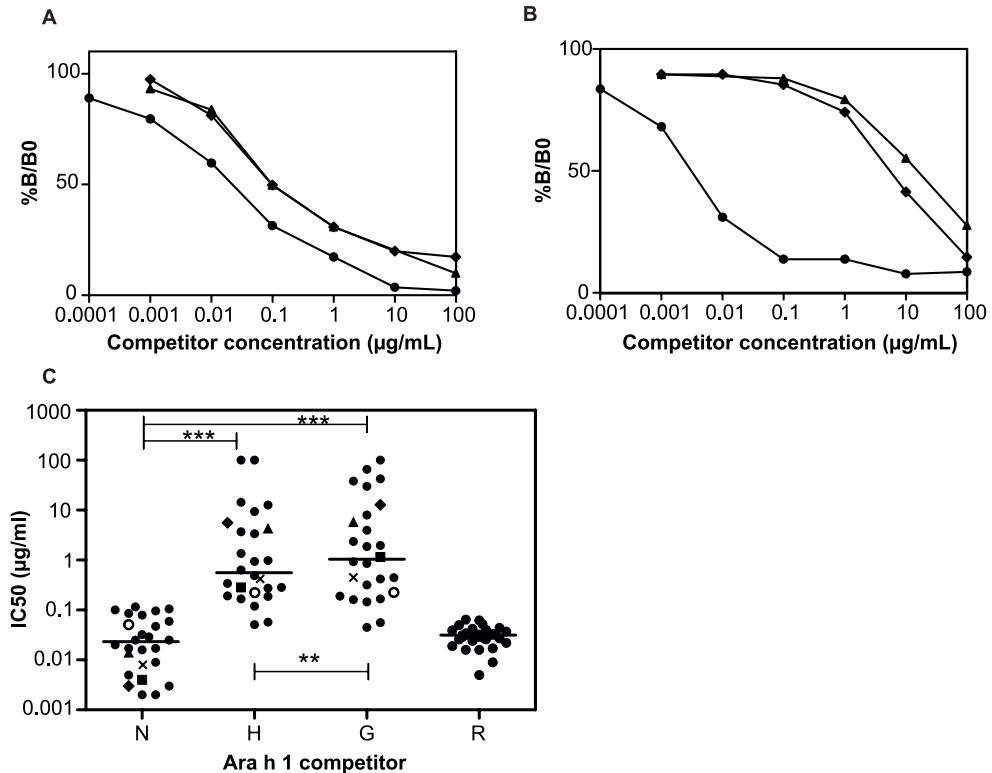


Fig. 4. IgE binding capacity of native and heat-treated Ara h 1. **A, B:** IgE capture inhibition curves obtained for serum #2205 and serum #66 respectively with N-Ara h 1 (●), H-Ara h 1 (◆) or G-Ara h 1 (▲). **C.** Analysis of IC₅₀ (ng/ml) values obtained using IgE capture inhibition assay with N-, H- G- and R-Ara h 1 as competitors and labeled N-Ara h 1 as a tracer (similar results were obtained when using labeled R-Ara h 1 as a tracer, data not shown). A serum panel from 26 peanut allergic patients was used with a reverse EAST inhibition assay and horizontal bars represents the median values of these patients. IC₅₀ values are inversely proportional to IgE-binding capacity. Bars indicate a significant difference between the 2 corresponding Ara h 1 samples determined using a non-parametric Wilcoxon signed rank test. *0.01 < *P* < 0.05, **0.001 < *P* < 0.01, ****P* < 0.0001. Individual symbols represent serum samples from individuals #55 (○), #65 (■), #66 (◆), #70 (▲) and #73 (x) also used in the RBL assay and who also donated PBMC samples.

Similar patterns of reactivity were observed when following β-hexosaminidase release with a humanized RBL cell line passively sensitized with sera from six patients (Table S1; Fig. 4B and 5A, serum #66). However, the loss of reactivity of the H- and G-Ara h 1 was smaller than that

observed by EAST, the EC₅₀ values being increased 6.9 and 7.5-fold respectively for H-Ara h 1 and G-Ara h 1 compared to N-Ara h 1 (Fig. 5B).

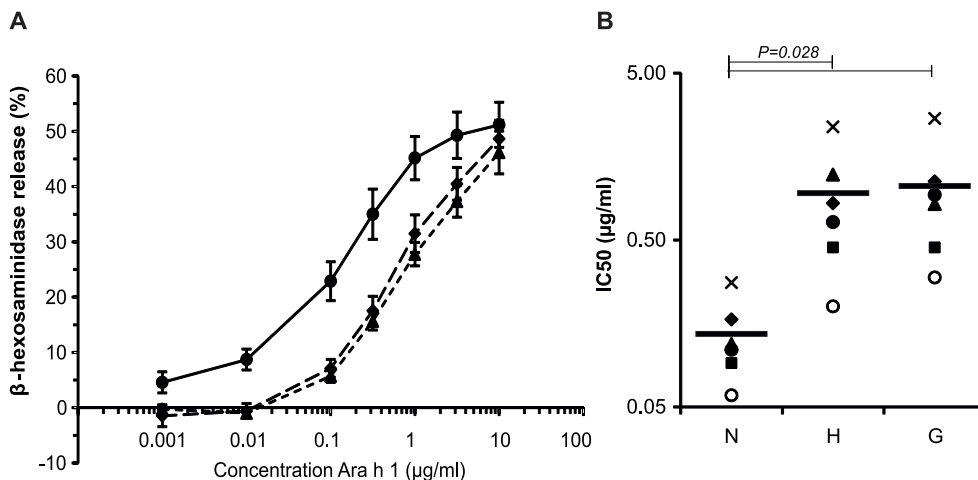


Fig. 5. Effect of thermal treatment on β -hexosaminidase release from humanized rat basophilic leukaemia (RBL) cells. **A.** Humanized RBL-2H3 cells were passively sensitized with sera from peanut allergic patients (serum #66) and stimulated with increasing concentrations of native (N; —●—), heated (H; --◆--) or glycosylated (G; ---▲---) Ara h 1. Error bars represent the SD of triplicate values. No β -hexosaminidase release was induced by the different allergens when using sera from non-allergic patients (data not shown). **B.** IC₅₀ values ($\mu\text{g/ml}$) obtained by MRA using 6 sera from peanut-allergic patients: #55 (○), #65 (■), #66 (◆), #67 (●), #70 (▲), and #73 (x). Horizontal bars represent the means of the six PA subjects. Bars indicate a significant difference between the 2 corresponding treatments.

In addition, the impact of the thermal modifications of Ara h 1 on the T-cell responses of PBMC from peanut allergic patients were assessed. Multicolour FACS-staining assessing surface marker expression in combination with intracellular Ki-67 expression as a sensitive method to detect antigen-specific proliferation [18], enabled us to measure subsets of proliferating cells. Stimulation of PBMC cultures from NA controls by Ara h 1 did not induce significant proliferation ($2.9 \pm 0.8\%$ and $1.9 \pm 0.5\%$ for unstimulated (medium) and allergen-stimulated (Ara h 1) cultures, respectively; Fig. 6A). Only three of the 12 PA subjects showed detectable numbers of Ki-67+ proliferating cells in the Ara h 1 stimulated cultures (subjects #65, #66 and #70; Fig. 6A) with a corresponding mean percentage of Ki-67+ cells of 4.7 ± 2.7 for medium and $11.9 \pm 3.4\%$ for the Ara h 1 stimulation. Proliferating cells consisted of 81% CD4+ cells and the CD4+CD25+ subset of cells was the largest cell population present in the proliferating cell fraction. For these three PA subjects, thermal treatment of N-Ara h 1 had no effect on its capacity to induce PBMC proliferation (Fig. 6B). Whilst no cytokine production was induced by Ara h 1 in PBMC cultures from the NA subjects (Fig. 6C, D; and data not shown), the production of IL-5 and IL-13 was significantly enhanced in the culture of PBMC from 5 out of 12 PA subjects (patients #65, #66, #67, #70 and #73) upon stimulation with N-Ara h 1. Heating in the presence or absence of glucose had no effect on these cytokine secretions. In PA subjects, an enhanced production

(compared to NA subjects) was also observed for IL-10 and IFN- γ , albeit not significant for all stimuli tested (data not shown). IL-17 production was below 20 pg/ml for all tested conditions (data not shown).

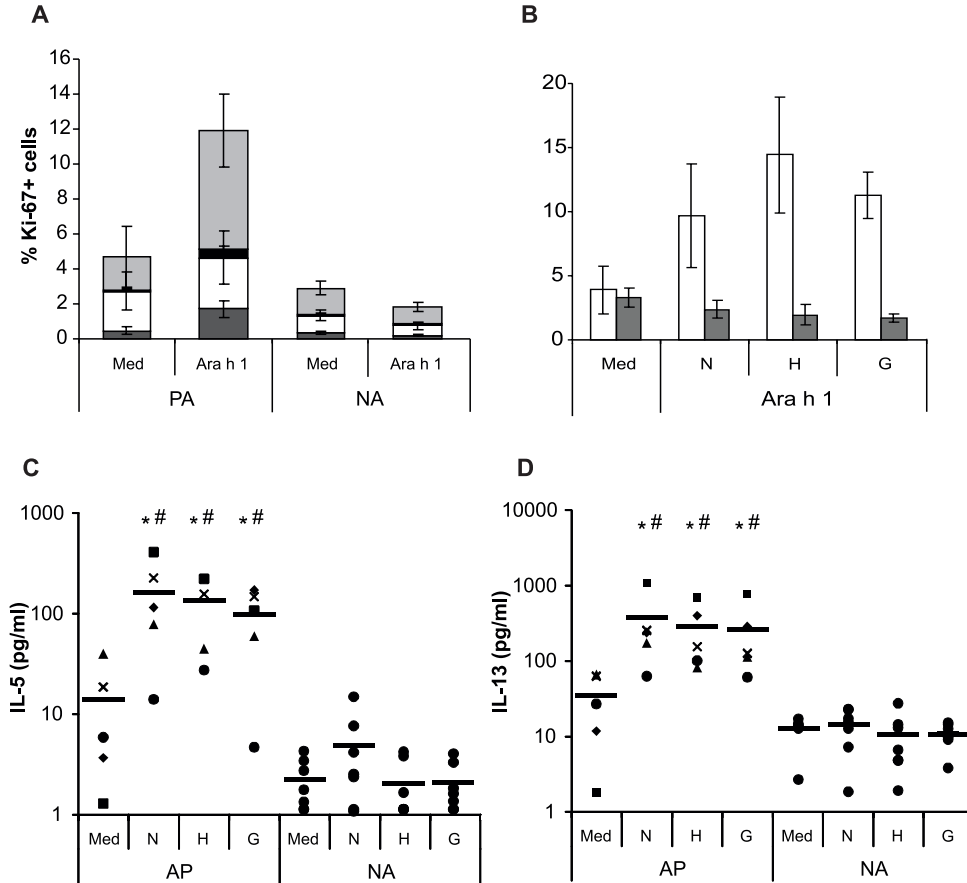


Fig. 6. Effect of thermal treatment on subpopulation of proliferating cells and cytokine induction capacity. **A.** Fresh PBMC were left unstimulated (Med) or stimulated with N-Ara h 1 for 7 days. After harvesting, the cells were incubated with anti-CD4/CD25 antibody and afterwards stained for an anti-Ki-67 PE antibody. Data are mean percentage of Ki-67 positive cells \pm SEM values for three (#65, #66 and #70) of the 12 PA subjects and seven NA controls. The percentage Ki-67+ cells is divided in the following subpopulations of cells: CD4-CD25- (dark grey bars), CD4+CD25- (white bars), CD4-CD25+ (black bars) and CD4+CD25+ (light grey bars) cells. **B.** Fresh PBMC were left unstimulated (Med) or stimulated with N-, H- or G-Ara h 1 for 7 days. Data are mean percentage of Ki-67 positive cells \pm SEM values for three peanut-allergic patients (#65, #66 and #70) (open bars) and seven non-allergic controls (closed bars). Fresh PBMC were left unstimulated (Med) or stimulated with N-, H- or G- Ara h 1 and production of IL-5 (**C**) and IL13 (**D**), were measured after 7 days of culture. Symbols represent peanut-allergic patients #65 (■), #66 (◆), #70 (▲), #67 (●) and #73 (x). Horizontal bars represent the means of the five PA subjects or the 7 NA controls. Asterisks indicate statistically significant differences between the PA and the NA group and # indicate statistically significant difference between the medium and allergen-stimulated cultures for the PA group (*, # $P < 0.05$). No dif-

ferences between the medium and allergen-stimulated cultures were observed for the NA group.

Discussion

We have sought to overcome the poor solubility and stability of thermally unfolded and aggregated proteins [2,9,19] by using a combination of model processing of purified allergens and purification of allergens from thermally-processed peanuts. For the first time we have shown that boiled Ara h 1 forms complex branched aggregates which have a much reduced IgE-binding capacity. The aggregates also had a reduced capacity to elicit histamine release, a measure of functional biological activity more indicative of a potential reduced allergenic potency *in vivo*. However, this reduction was not as marked as might be expected given the reduction in IgE-binding capacity, and may reflect the likely multiple IgE epitopes present on the aggregates which might be more efficient in cross-linking surface bound IgE. Further studies *in vivo* will be required to confirm the potential clinical relevance of this observation.

By comparison, Ara h 1 purified from roasted peanuts formed aggregates which were smaller and morphologically distinct from those induced by boiling and appeared to retain more native-like β -sheet structures by FT-IR than the boiled protein. Such structural features account for R-Ara h 1 retaining its allergenic IgE-binding capacity, and is consistent with previous observations that Ara h 1 purified from peanuts heated up to 140°C retained its IgE binding capacity [9]. These data suggest aggregate morphology could play a role in affecting IgE binding by altering Ara h 1 epitope availability, a greater proportion of epitopes being masked in aggregates formed by boiling of Ara h 1, compared to the smaller, less complex aggregates adopted by the R-Ara h 1 from roasted peanuts. The formation of branched aggregates with reduced IgE binding capacity following boiling supports the proposition that boiling reduces the allergenicity of peanut kernels and suggests that many Ara h 1 epitopes are found on the surface of the native proteins and are conformational in nature [2].

The ability of Ara h 1 to retain, or even have enhanced allergenicity following roasting, has also been attributed to Maillard modifications. However the R-Ara h 1 was not as extensively glycosylated as the model processed G-Ara h 1 suggesting that roasting may cause other, uncharacterized modifications of the protein which contribute to its allergenic activity. This may include modification of amino acid residues, such as lysoalanine formation, or adducts formed with other peanut constituents such as lipids. Further characterization of processing induced modification of this protein from roasted peanuts will be required to fully understand what contributes to the ability of this protein to retain its allergenic activity after processing.

In contrast, the ability of Ara h 1 to simulate the proliferative activity of PBMC from peanut allergic patients was unaltered following heat treatment and/or glycation, as has been shown before for Bet v 1-related allergens [20,21] and for heat-treated Ara h 2/6 (Vissers *et al.*, submitted). The peanut allergic patients who showed an Ara h 1 specific T-cell response in this study also showed an Ara h 2/6 specific T-cell response and were all characterized by high peanut specific IgE. The magnitude of the stimulation, in terms of proliferation and IL-5 and IL-13 cytokine induction, was comparable for Ara h 1 and Ara h 2/6 (Vissers *et al.*, submitted). This suggests that peanut allergic individuals who have high peanut-specific IgE titers have a higher prevalence of allergen-specific T cells and therefore give the most pronounced responses in

primary PBMC cultures. However, Turcanu *et al.* observed that peanut-specific T-helper cells in PBMC of peanut allergic patients were 0.6% of the total CD4+ T cells in peanut allergic patients and about 10 times lower in non-allergic and peanut sensitized donors [22]. Altogether these results may explain the low rate of responders in our *ex vivo* stimulation assays.

Studies of other allergens, particularly in the search for hypoallergenic allergens support the view that aggregation of proteins may affect the quality of immune responses. Recent studies having shown that for the Bet v 1 allergens, aggregation may play an important role in establishing protective antibody titers in immunotherapy [23]. The aggregates produced by boiling Ara h 1 presented here resemble the properties of allergoids which have been well accepted for immunotherapy for many years and tend to have lower IgE reactivity, whilst retaining their ability to activate T-cells [24]. Whilst boiling for 15 min did not completely abrogate IgE reactivity there is potential to reduce it further through application of more longer or more severe wet-heating regimes. Our results suggest that aggregated forms of allergens induced by physical processes like boiling may provide a novel form of antigen for use in allergen-specific immunotherapy as well as supporting the premise that boiling, as opposed to roasting, may reduce the allergenicity of peanut.

Acknowledgements

We wish to thank Kurt Quartz for organization of the trial within Rijnstate Hospital, the Netherlands. The work described was carried out under the EU FP6 project “The Prevalence, Cost and Basis of Food Allergy Across Europe”, EuroPrevall (Contract No. 514000). ENCM, PEJ and NR were also supported by the institute strategic program grant to IFR from the Biological and Biotechnological Sciences Research Council in the UK.

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Supplement file

Table S1. Serological and clinical information for the 35 peanut allergic patients recruited in different European centers, within the EuroPrevall European project, used in EAST studies (bold), RBL tests (*) and/or PBMC proliferation tests (#).

Code	Gender (f/m)	Age	Peanut SPT (HEP Index numbers)	Grade peanut allergy	Peanut RAST class	Peanut CAP (IU/mL)	Other food allergies
2A	m	31	positive	3/4	5	51.7	
52A	m	41	nd	4	5	nd	
54A	f	11	2.2	1	6	>100	
55A*	f	14	3.3	4	6	>100	
56A	m	16	1.5	2	6	>100	
58A	f	22	2.5	4	6	>100	Soy, Lupine, Pea
65*#	f	19	positive	3/4	6	>100	
66*#	m	18	positive	4	6	>100	
67*#	f	46	nd	3	5	54.4	Hazelnut, Kiwi, Cherries
68#	f	20	positive	1	4	17.6	
69#	m	21	positive	3/4	3	6.98	
70*#	f	21	positive	3/4	6	>100	Soy, Pea, Apple
73*#	f	22	positive	2/3	4	33.6	Hazelnut
76#	m	23	positive	3	3	8.6	
77#	m	25	positive	2	4	33.2	
78#	f	24	positive	3	5	79.6	Hazelnut
80#	m	27	positive	3	3	16	
82#	f	18	positive	1/2	3	4.7	Kiwi, Cherries
1682	m	33	nd	4	6	>100	
1691	m	22	nd	3	5	nd	
2205	m	25	nd	3	5	nd	Hazelnut
2207	m	28	nd	4	4	nd	Wheat, Hazelnut, Egg, Almond
2209	f	27	nd	4	6	nd	Hazelnut, Egg, Wheat
2304	m	25	nd	3	6	nd	
2305	f	18	nd	4	nd	nd	
03-0043	f	20	1.54	3/4	6	>100	Hazelnut, Walnut, Melon
03-0044	m	23	4.33	3	6	>100	Egg, Fish, Shrimp, Hazelnut, Walnut, Sesame, Wheat, Sunflower
03-0079	f	16	2.22	3	6	>100	Pistachio, Almond, Brazil nut
05-0128	f	25	1.92	3	6	>100	Soy, Hazelnut, Apple, Peach
05-0165	f	18	0	3/4	6	>100	Chickpea, Bean, Hazelnut, Lentil
05-0195	m	31	2.13	3	5	63.5	Soy
05-0209	m	20	99.98	4	5	70.2	Pea, Soy, Bean
06-0010	m	19	2.2	3/4	5	67.4	Fish
08-0069	m	17	2.08	1	6	>100	Kiwi
12-0048	f	21	5.8	3/4	5	54.6	Shrimp

HEP: histamine equivalent prick test; EPSB: EuroPrevall Serum Bank; nd: not determined; Where known, other allergies of the subjects are indicated. The origin of the sera is as follows: 2A: Zurich, 52A: Amsterdam, 54A-82: Arnhem, 1682-2305: Vienna; 03-0043 till 12-0048 EuroPrevall Serum Bank.

Grading is based on Brockow and Ring and on Sampson *et al.* [25,26]. Grade 1 = dermal symptoms; grade 2 = gastro-intestinal problems like nausea and or cramping; grade 3 = any of the former grades plus vomiting/diarrhea

and respiratory tract problems like throat pruritus or tightness; grade 4 = any of the former grades and respiratory arrest plus cardiovascular problems like hypotension

Directly after isolation of the PBMC from the 12 peanut allergic (PA) and 12 non allergic (NA) donors, a surface marker staining showed that the subset composition of the peanut-allergic patients was comparable to the subset composition of cells of the non-allergic donors. All values were within the normal range of leukocytes present in the peripheral blood as assessed previously [27,28]. Median percentages and ranges (percentile 5-95) of cell subsets within the PBMC population for the 24 subjects were as follows: 39% (25-57) CD3+CD4+, 20% (8-35) CD3+CD8+, 13% (6-26) CD14+, 8% (4-11) CD19+, 9% (6-14) CD16+CD56+CD3-, 4% (1-9) CD16+CD56+CD3+ cells. The average percentage of viable and early apoptotic lymphocytes directly after isolation was $92\pm 1\%$ and $8\pm 1\%$, respectively (data not shown). To exclude possible toxic effects of the allergen preparations tested and to ensure the viability of the cells of the long-term cultures, an Annexin V/PI staining was performed after 7 days of allergen-stimulated cultures and of the unstimulated control. No difference was observed between the different stimuli and the medium, or between the PA subjects and the NA controls. On average $84\pm 1\%$ of the cells was regarded as viable cells.

After 4 days of culture, proliferation was measured on both unstimulated and α CD3/ α CD28-stimulated cultures to assess the maximal intrinsic capacity of the T cells of both tested groups to respond to a stimulus. Without stimulus the percentage of Ki-67 positive cells was below 8% for all 24 patients tested. The average percentage of Ki-67 positive cells of α CD3/ α CD28-stimulated cultures was $58.3\pm 3.9\%$ for the PA subjects and $54.0\pm 4.6\%$ for the NA controls, which was not significantly different (Fig. S1). For the 24 subjects tested, the proliferating cell population after the α CD3/ α CD28-stimulation consisted of 97% CD25+ cells and 48% were CD4 positive (Fig. S1). In the unstimulated cultures, IL-5, IFN- γ and IL-17 levels were below 20 pg/ml for all patients tested and below 50 pg/ml for IL-10 and IL-13. After α CD3/ α CD28-stimulation, cytokine levels of the PA subjects versus the NA controls were respectively IL-10= 675 ± 155 vs 905 ± 216 , IFN- γ = 6654 ± 1242 vs 7925 ± 1106 , IL-17= 209 ± 94 vs 134 ± 12 , IL-13= 479 ± 113 vs 514 ± 226 and IL-5= 64 ± 20 vs 251 ± 215 . Due to high inter-individual variation, these cytokine values did not significantly differ between the two donor-groups tested.

The analysis of proliferation and cytokine production after polyclonal α CD3/ α CD28 stimulation thus revealed that the T-cell compartments in the NA and PA subjects did not show intrinsic differences in their activation potential and characteristics

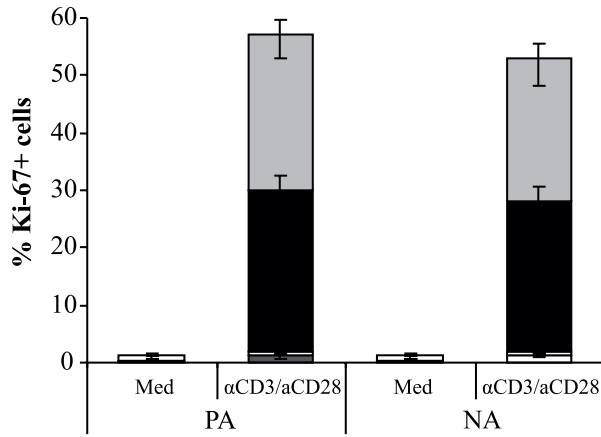


Fig. S1. Fresh PBMC were left unstimulated (Med) or stimulated with α CD3/ α CD28 for 4 days. After harvesting, the cells were incubated with anti-CD4/CD25 antibody and afterwards stained for an anti-Ki-67 PE antibody. Data are mean percentage of Ki-67 positive cells \pm SEM values for 12 peanut allergic (PA) and 12 non allergic (NA) controls. The percentage Ki-67+ cells is divided in the following subpopulations of cells: CD4-CD25- (dark grey bars), CD4+CD25- (white bars), CD4-CD25+ (black bars) and CD4+CD25+ (light grey bars) cells.

Chapter 4

Effect of roasting on the allergenicity of major peanut allergens Ara h 1 and Ara h 2/6: the necessity of degranulation assays

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Submitted paper

Summary

Background: Peanuts are often consumed after roasting, a process which alters the three-dimensional structure of allergens and leads to Maillard modification. Such changes are likely to affect their allergenicity.

Objective: We aimed to establish the effect of thermal treatment mimicking the roasting process on the allergenicity of Ara h 1 and a mix of 2S albumins from peanut (Ara h 2/6).

Methods: Ara h 1 and Ara h 2/6 were purified from raw peanuts and heated in a dry form for 20 min at 145°C in the presence (R+g) or absence (R-g) of glucose, and soluble proteins were then extracted. Sera obtained from 12 well-characterized peanut-allergic patients were used to assess the IgE binding and degranulation capacities of the allergens.

Results: Extensive heating at low moisture resulted in hydrolysis of both Ara h 1 and Ara h 2/6. However, in contrast to Ara h 2/6, soluble R+g Ara h 1, formed large aggregates. Although the IgE binding capacity of R+g and R-g Ara h 1 was decreased 9000- and 3.6-fold respectively compared with native Ara h 1, their capacity to elicit mediator release was increased. Conversely, both IgE binding capacity and degranulation capacity of R-g Ara h 2/6 were 600-700-fold lower compared with the native form, although the presence of glucose during heating significantly moderated these losses.

Conclusions and Clinical Relevance: Extensive heating reduced degranulation capacity of Ara h 2/6 but significantly increased the degranulation capacity of Ara h 1. This observation can have important ramifications for component resolved approaches for diagnosis and demonstrates the importance of investigating the degranulation capacity in addition to IgE reactivity when assessing effects of food processing on the allergenicity of proteins.

Introduction

Peanut allergy is a common food allergy in the USA and in European countries affecting around 1% of the population [1,2]. In some studies the prevalence was found to be much higher, with approximately 10% of 8-year-old children in the UK being sensitised to peanut, of whom 2% have a clinical peanut allergy [3]. **In addition, the prevalence of peanut allergy in children appears to be increasing [2], which contributes to a growing global concern, particularly given the severity of peanut-allergic reactions.**

Recent studies indicate that peanut-allergic patients are most frequently sensitized to Ara h 2 and Ara h 6, followed by Ara h 1, Ara h 3 and Ara h 7 [4,5]. Ara h 6 and Ara h 7 show around 55% and 40% amino acid sequence homology with Ara h 2, respectively [6,7], suggesting that these three proteins could share a number of identical epitopes [6,8]. **However, epitopes specific to Ara h 2 and Ara h 6 also exist [4].** Ara h 2, 6 and 7 all belong to the family of the 2S albumin storage proteins and have a molecular weight between 16 and 20 kDa [6]. Ara h 1 is a vicilin-like 7S seed storage globulin, also known as conarachin. Ara h 1, comprising 63.5 kDa subunits that assemble naturally into trimers of approximately 180 kDa, often aggregates forming multimers of up to 600-700 kDa [9-11]. Twenty-three linear IgE epitopes have been identified within Ara h 1, mainly in the areas of the subunit-subunit contacts that are protected

from protease degradation [12-14].

Food processing can potentially destroy or conversely create linear and conformational IgE binding epitopes, establish the exposure of formerly hidden antigenic sites, and change the susceptibility to digestion [15]. These changes may affect allergic sensitization to food proteins and elicitation of allergic reactions in sensitised individuals thereby impacting on the allergenic potential of the food proteins. Important modifications that food proteins may undergo are observed as a consequence of the Maillard reaction, which involves the reaction of free amino groups on proteins (particularly lysine residues) with reducing sugars, thereby inducing flavour and colour of processed foods including peanuts [16,17]. The extent of glycation depends on the temperature, pH, water activity, duration of the heating and the concentration of the reducing sugars present [18]. Whilst the Maillard reaction can affect the allergenicity of peanut proteins, this has not been conclusively demonstrated and depends on the type of protein and the conditions under which the Maillard reaction occurred. In addition, the type and amount of carbohydrate used and other matrix compounds present during heating also affect allergenicity [16,17,19-23].

The effect of processing on the allergenicity of proteins has been assessed by determining the IgE binding capacity using immunoassays and/or immunoblotting. However, the impact of food processing on the cross-linking and degranulation capacity is important, since processing-induced modifications are likely to affect the availability of the multiple epitopes required for effective cross-linking of cell surface bound IgE. In the current study, the effects of processing were also assessed using two cellular mediator release assays to determine allergen cross-linking capacity of IgE antibodies bound to FcεRI of mast cells/basophils. Roasting induces an array of protein modifications due to interaction with the food matrix, affecting the purification efficacy of the modified allergens. Therefore, the allergens were first purified from untreated peanut, lyophilized and then subjected to a model thermal treatment mimicking the roasting process.

Materials and Methods

Patients

Peanut-allergic patients from the Allergology Practice Arnhem (APA, the Netherlands) were approached to participate via an invitation letter. Written informed consent was obtained before sample collection and the experiments performed were approved by the local ethical committee (Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen). The studied population included 12 peanut-allergic patients. All patients had a well-documented peanut allergy based on an extensive medical history, physical examination, and objective clinical manifestations observed after peanut ingestion. Grading of food-induced anaphylaxis was according to severity of clinical symptoms [24,25]. From all patients, except one, a skin prick test with commercial peanut extract (ALK-Abelló, Nieuwegein, the Netherlands) was performed and peanut-specific IgE levels were determined using ImmunoCAP (Phadia AB, Uppsala, Sweden).

Isolation of Ara h 1 and Ara h 2/6

Native Ara h 1 (N-Ara h 1) was purified from redskin type raw peanuts (obtained from local suppliers, UK) as described by Marsh *et al.* [26]. The purification is based on affinity chromatography using concanavalin A-Sepharose followed by gel permeation chromatography using a Superdex Preparative Grade S200 column (GE Healthcare, Buckinghamshire, UK). Protein preparations were adjudged >99% pure by SDS-PAGE.

Native 2S albumins containing both Ara h 2 and Ara h 6 (N-Ara h 2/6) were purified from unroasted redskin type peanuts as described previously and allergen identity was confirmed by in gel-trypsin digestion and MALDI-TOF mass spectrometry [27]. This purification involved ammonium sulphate fractionation and gel-filtration chromatography, avoiding the use of denaturing conditions such as reverse phase HPLC. Purified native proteins were stored at -20°C prior to use.

Roasting treatment of the purified allergens

Purified native Ara h 1 in 32.5 mM phosphate buffer (pH 7.6) containing 100 mM NaCl and purified native Ara h 2/6 in 25 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.02% (w/v) NaN_3 were split in three batches. One batch of the protein was kept untreated (native form). One batch was mixed with glucose (Sigma-Aldrich, Steinheim, Germany) in a ratio of 1:2 (protein:glucose, w/w) in PBS. The batch with glucose and one batch without glucose were placed in glass bottles, frozen at -70°C and lyophilized. Lyophilized samples were then heated in a forced convection Chamber of Thermal Research (Wamed, KBC-65W) under conditions chosen according to Saklar *et al.* [28]. **After establishing a steady temperature of 145°C, the proteins were heated for 20 min and then samples were allowed to cool to ambient temperature.** Samples were dissolved in Milli-Q water, centrifuged (5000×g, 15 min, 4°C) and the supernatant collected and ultrafiltered by Amicon Ultra-15 centrifugal filters (10,000 nominal molecular weight limit (NMWL) for Ara h 1 and 5,000 NMWL for Ara h 2/6) (Millipore, Billerica, MA, USA) against PBS for several rounds to remove the residual glucose, the hydrophilic glucose degradation products from the glycosylated proteins and other small molecular weight products. By this, the allergenic activity of the protein itself, and not of other compounds formed during roasting, was assessed. Finally, the protein concentration was assessed by the BCA-assay and aliquots were stored at -70°C. Samples will be referred to as N (native), R+g (heated at low moisture in the presence of glucose) or R-g (heated at low moisture in the absence of glucose).

Biochemical characterization

SDS-PAGE and Western blot analysis

SDS-PAGE (12%) was performed under reducing conditions on a mini-protean II gel apparatus (Bio-Rad, Veenendaal, the Netherlands) [29]. The gels were stained using Gel Code blue stain (Pierce, Rockford, IL, USA) and the precision plus protein dual colour MWM was used as the molecular weight standard (Bio-Rad).

For analysis of IgE-binding fragments of the thermally processed Ara h 1 and Ara h 2/6, a Western blot analysis was performed using a plasma pool of the first nine peanut-allergic patients described in Table 1. SDS-PAGE (12%)-separated proteins were electrophoretically transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The

membranes were blocked in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris, 500 mM NaCl, 0.05% (v/v) Tween, pH 7.5) plus 3% BSA for 1 h at room temperature and then incubated with a 1:15 dilution of the pooled human plasma overnight at 4°C in 0.5% BSA in TBST. After incubation for 1 h with biotin labelled mouse anti-human IgE (1:250, BD Pharmingen, San Diego, CA, USA) in TBST plus 5% non-fat dry milk, membranes were washed and bound IgE was detected by streptavidin poly-horse radish peroxidase (1:4000, Sanquin, Amsterdam, the Netherlands) in TBST plus 5% milk powder for 1 h at room temperature. Between each incubation step, membranes were washed twice with TBST/Triton (TBST, 0.2% (v/v) Triton X-100) and once with TBST, for 10 min at room temperature. Signal was detected by development with a chemiluminescence kit (Amersham Biosciences, Roosendaal, the Netherlands) according to the manufacturer's protocol and visualized by the use of Lumi-film chemiluminescent Detection Film (Roche, Mannheim, Germany).

Protein size measurement by light scattering

The measurements of the average size were carried out on a Malvern Zetasizer Nano-ZS analyser (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Dynamic light scattering (DLS) was performed and the intensity of the light scattered by the protein was measured at an angle of 173°. Samples were measured in disposable micro cuvettes (Malvern) at a concentration of 250 µg/mL in sterile PBS, pH 7.4. First, the sample was equilibrated for 300 seconds at 20°C after which a time scale of the scattered light intensity fluctuations was measured. Every sample was measured in triplicate and autocorrelation analysis was carried out with the use of the Zetasizer software for the Nano, v6.20 (Malvern Instruments Ltd). Data was processed using multiple narrow modes (high resolution) as analysis model, which gave the lowest multimodal fit error. Data are presented both as size distribution by volume as by intensity.

o-Phthaldialdehyde (OPA) Assay

The OPA assay was applied to determine the quantity of free amino groups according to Nielsen *et al.* [30] with slight modifications. The protein solution (250 µg/ml) was mixed with the reagent in a ratio of 1:10 (v/v) and incubated for 20 min at room temperature. The absorbance was measured at 340 nm and samples were measured in triplicate. Unreacted amino groups were estimated from a calibration curve established with L-leucine.

Analysis of the IgE immunoreactivity

Concentrations of total IgE and of IgE specific to purified Ara h 1 and Ara h 2/6 were determined using the enzyme allergosorbent assay (EAST) as previously described [21,31].

Enzymatic Ara h 1 tracer was prepared by covalent linkage of N-Ara h 1 to the tetrameric form of acetylcholinesterase (AChE using N-succinimidyl-S-acetyl-thioacetate (SATA) reacting with maleimido groups previously incorporated into AChE with N-succinimidyl-4-(maleidomethyl)-cyclohexane-1-carboxylate (SMCC) as described [32,33]. Native Ara h 2/6 was dialyzed against borate buffer 0.1 M pH 9 (NMWL 3500 Da), and then incubated for 45 min at room temperature with EZ-link sulfo-NHS-LC biotin (Pierce, Rockford, IL, USA) using a 10:1 molecular ratio biotin:N-Ara h 2/6. Biotinylation was stopped by further incubation for 1 h in 1 M Tris buffer, pH 8.5. Biotinylated N-Ara h 2/6 were then dialyzed and diluted in EIA buffer and kept at 4°C until use.

Inhibitions were performed in a reverse EAST [32]. Briefly, 50 μL /well of different dilutions of each serum which were optimized in an initial experiment were distributed on plates coated with the anti-human IgE (mouse monoclonal, clone LE27; described by Grassi *et al.* [34]) and incubated overnight at 4°C. After washing, 25 μL of inhibitor and 25 μL of tracers were added and incubated for 4 h at room temperature. Inhibitors consisted of increasing concentrations of N-, R+g or R-g Ara h 1 (10^{-4} – 100 $\mu\text{g}/\text{mL}$) or Ara h 2/6 (10^{-5} – 10 $\mu\text{g}/\text{mL}$). Enzymatic tracers consisted of N-Ara h 1 labelled with AChE or biotinylated N-Ara h 2/6 (200 ng/mL). When using a biotinylated tracer, an additional 45 min incubation with 50 μL of streptavidin labelled with AChE was performed. After washing, Ellman's reagent was used as an enzyme substrate [35]. Results were expressed as B/B_0 , where B_0 and B represent the amount of N-Ara h 1 or N-Ara h 2/6 tracers linked to immobilized IgE in the absence or presence of a known concentration of inhibitor, respectively.

Indirect basophil histamine release (BHR)

Buffy coats were obtained from the hospital blood bank in Copenhagen and cells were screened and selected for a high anti-IgE response and no allergies. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep gradient centrifugation, cell-bound IgE was stripped from the cells by a phosphate buffer and BHR tests were performed as described previously [36]. Six dilutions (0.001 – 100 $\mu\text{g}/\text{mL}$) were tested for Ara h 1 and 12 (0.0002 – 20 $\mu\text{g}/\text{mL}$) for Ara h 2/6 and the concentration range chosen was based on earlier optimization experiments. Allergens were graded based on the lowest concentration inducing a histamine release (HR) of > 10 ng/mL, in which grade 1 is basophils only reacting to the highest concentration of the allergen tested, and 6 is basophil reaction to the lowest concentration tested.

Mediator release assay (MRA) using Rat Basophilic Leukemia (RBL) cells

RBL-2H3 cells expressing the α -chain of the human Fc ϵ RI receptor [37] were kindly provided by Drs. Vieths and Vogel (Paul-Ehrlich-Institut, Langen, Germany). In a preliminary experiment, four sera dilutions (1:20, 1:30, 1:40 and 1:50) and three concentrations of a peanut extract (0.01, 0.1 and 1 $\mu\text{g}/\text{mL}$) were tested for all 12 sera and suitable sera were selected based on the percentage β -hexosaminidase release. Cells were cultured in MEM medium supplemented with 5% FCS and 1% glutamine (all from Gibco, Paisley, UK) at 37°C in a humidified atmosphere with 5% CO₂. Cells in stationary growth phase (confluent or slightly overgrown) were harvested and plated in 96-well plates at 1.5×10^5 cells/well. Human sera (1:30) were added and incubated overnight to passively sensitize the cells. After washing, the cells were stimulated for 1 h with the allergens diluted in Tyrode's buffer containing 50% deuterium oxide [37]. The antigen-specific release was quantified by measuring β -hexosaminidase activity and expressed as percentage of the total β -hexosaminidase content that was obtained by lysing the cells with 1% Triton-X100 (Sigma-Aldrich, Zwijndrecht, the Netherlands) [38]. Spontaneous release was determined on cells not sensitized nor cross-linked. Toxicity of each serum was also checked using sensitized cells not further cross-linked with allergens. The release data were fitted to four-parameter-logistic curves by non-linear regression using the SigmaPlot v10.0 software package, and IC50 values were assessed accordingly.

Statistical analysis

Due to a non-normal distribution of the data, the nonparametric Wilcoxon signed rank test was used to compare IC₅₀ values and allergen classes between the native and treated allergens. The difference was considered statistically significant for $P < 0.05$. The statistical analysis was performed by using SPSS Software (v15.0, SPSS Inc., Chicago, USA).

Results

Effect of model thermal treatment on allergen structure

Blanched kernels of peanuts are subjected to oil roasting for around 5-8 min at 149-163°C for optimum flavour and colour [39], a process which results in extensive, but not complete, denaturation of peanut kernel proteins. In order to mimic this process, purified proteins were freeze dried to reduce the moisture content to about 4-5% [40], which corresponds to the moisture content of blanched peanuts. Inclusion of sugar in the samples prior to freeze-drying resulted in a further reduction of the moisture content, to about 1.5-3% [40].

Samples heated in the presence of glucose turned brown due to the formation of Maillard browning products. Soluble proteins were recovered from the thermally-treated proteins and this yield differed for the individual proteins. Around 75% of the Ara h 1 and 53% of the Ara h 2/6 remained soluble after heating without glucose. The addition of glucose resulted in a dramatic loss of soluble protein for Ara h 1, with only 5% of the protein being recovered. Ara h 2/6 solubility (58%) was very similar to that of the sample heated without glucose (data not shown).

Analysis of the residual soluble Ara h 1 showed that heating had resulted in significant hydrolysis of the protein (Fig. 1A), with appearance of two new polypeptides of about 23 and 33 kDa and substantial material of < 15 kDa appearing at the gel dye front after heating in the absence of glucose. This was accompanied by a loss of the parent 63.5 kDa polypeptide. The faint smeared appearance is indicative of some level of polypeptide aggregation and the formation of numerous heterogeneous structures due to different stages of the Maillard reaction. Addition of glucose during the heating resulted in a loss of any polypeptides resolvable by SDS-PAGE with all the stained proteins having a Mr < 15 kDa and a faint smear present in the protein stained gel, suggesting that heating in the presence of glucose had resulted in more extensive degradation. However, much of the protein did not enter the SDS-PAGE due to large aggregate formation as substantiated by the light scattering results (see below). The degradation products in both samples were still able to bind IgE antibodies from the peanut-allergic patients in the IgE-immunoblot experiment (Fig. 1C) despite R+g Ara h 1 being present at such low levels they were barely discernable on the protein stained gel (Fig. 1A). The thermal treatment also resulted in degradation of Ara h 2/6 to low molecular weight polypeptides below the resolving capacity of the SDS-PAGE gel used, with a substantial amount of protein-staining observed at the dye front of the gel (Fig. 1B). Heating in the presence of glucose resulted in the protein being poorly resolved with a smear evident in the protein stained gel. The pyrolysis products of the Ara h 2/6 were able to bind IgE, with individual polypeptides discernable on the Western blot (Fig. 1D) showing that Ara h 2/6 also retained its IgE binding capacity even after such extreme thermal treatment.

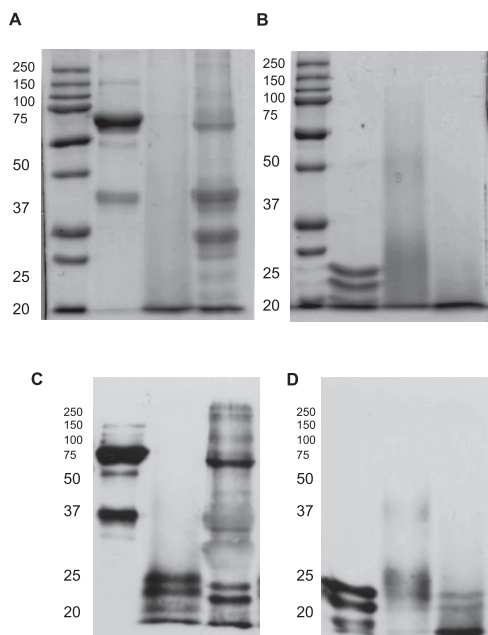


Fig. 1. SDS-PAGE and Western blot of the native and thermally processed Ara h 1 and Ara h 2/6 proteins. Ara h 1 (**A**) and Ara h 2/6 (**B**) were run on SDS-PAGE and stained with Coomassie blue stain. The same samples were transferred to nitrocellulose and analysed by Western blot analysis using a pool of plasma from 9 peanut-allergic patients. The bound plasma IgE was recognized by biotin labelled mouse anti-human IgE in combination with strep poly HRP and visualized by chemoluminescence (**C** for Ara h 1 and **D** for Ara h 2/6). MwM = molecular weight standard; R-g = heated in the absence of glucose; R+g = heated in the presence of glucose

The content of free amino groups was significantly lower in the heated Ara h 1 samples and lowest in the Ara h 1 heated in the presence of glucose as determined by the OPA assay (Table 2). For Ara h 2/6 roasting did not significantly change the free amino groups content significantly (data not shown).

The effect of thermal treatment on aggregation state of the soluble fraction of the Ara h 1 was also assessed using fixed angle dynamic light scattering (Table 2, Fig. 2). The unheated native Ara h 1 sample had a mean hydrodynamic diameter of around 15 nm, consistent with the protein being present in its native trimeric state. Heating alone resulted in the soluble fraction of Ara h 1 having a slightly reduced size of around 12 nm, consistent with the hydrolysis of the protein observed by SDS-PAGE although part of these degraded polypeptides were clearly assembled into much larger aggregated structures, ranging in size from 30 to 400 nm analysed by intensity (Fig. 2B). The soluble fraction remaining after heating in the presence of glucose was present as an ensemble of very large aggregated structures ranging from ~200 to 1000 nm in size, with an average diameter of 560 nm (Table 2, Fig. 2). Due to the relatively small dimensions of Ara h 2/6 this type of analysis was not performed for the 2S albumins.

Table 1. Biochemical analysis of native and heated allergens.

	N-Ara h 1	R+g Ara h 1	R-g Ara h 1
Percentage free primary amino groups in relation to the native allergen (mean SD)	100	16.1±1.4	35.1±3.6
Mean size ± width (d.nm)*	15.1±3.2	563±191	12.1±2.6
Zav (d.nm)**	16.2±0.1	389±13.3	45±0.5

* Analysed as size distribution by volume of the most common peak which represented for all samples >98% of the total volume. ** Z-average (Mean±SEM) of triplicate measurements; analysed as size distribution by intensity and including all peaks

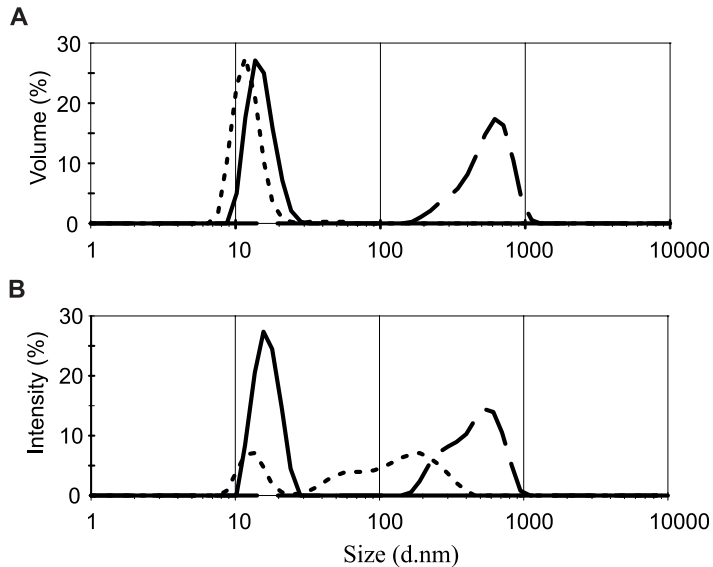


Fig. 2. Particle size distribution of N, R+g and R-g Ara h 1 measured with dynamic light scattering. Light scattering measurements of native (N; —), heated in the presence of glucose (R+g; - - -) or heated in the absence of glucose (R-g; · · · ·) Ara h 1 protein particles. Results are shown as size distribution by **A.** volume, percentage volume versus size in nm and **B.** intensity, percentage intensity versus size in nm. Data represent the mean of three measurements per sample.

Allergenic activity of thermally-modified peanut allergens

Nine of the 12 peanut-allergic patients (5 males and 7 females, median age: 22 years) showed grade 3 or 4 clinical peanut allergy and all except one a highly positive (++) SPT (Table 1). Specific IgE values for peanut extract, Ara h 1 and Ara h 2/6 were assessed in the sera of the patients (Table 1). All patients were polysensitized for the different peanut allergens tested (Ara h 1 and Ara h 2/6), which is in accordance with a recent study that showed only a low percentage (7%) of mono-allergen sensitized peanut-allergic patients [4].

Table 2. Serological and clinical information for the 12 peanut-allergic patients recruited from the Allergy Practice Arnhem, the Netherlands.

Code	Age (years)/sex (f/m)	Grade peanut allergy	SPT (-/+/+)	Other food allergies	Total IgE (kU/L)	Specific IgE (kU/L)		
						Peanut (f13)	Ara h 1	Ara h 2/6
65	19/f	3/4	++	no	787	>100	267	208
66	18/m	4	++	no	514	>100	173	71
70	21/f	3/4	++	soy, pea, apple	1869	>100	117	76
78	24/f	3	++	HN	406	79.6	62	29
67	46/f	3	nd	HN, kiwi, cherries	3986	54.4	51	35
73	22/f	3/4	++	HN	96	33.6	19	3.7
77	25/m	2	++	no	86	33.2	21	3.5
68	20/f	1	++	no	109	17.6	10	6
80	27/m	3	++	no	110	16	6.9	17
76	23/m	3	++	no	108	8.59	6	0.3
69	21/m	3/4	++	no	200	6.98	2.2	1.2
82	18/f	1/2	+	kiwi, cherries	23	4.75	2.3	6.4

IgE concentrations (IU/mL) of total IgE and IgE specific for Ara h 1 and Ara h 2/6 were determined by EAST assay

on anti-IgE or allergens coated plates. Each serum was tested as serial dilutions from 1/4 to 1/2500, in duplicates. Limits of detection of the assays were 0.1 IU/mL, permitting detection values > 100 kU/l. Specific IgE for peanut (f13) was determined using ImmunoCAP (Phadia AB) and values were capped at 100 kU/l. nd = not determined, HN = hazelnut

Grading is based on Brockow and Ring and on Sampson *et al.* [24, 25]. Grade 1 = dermal symptoms; grade 2 = gastro-intestinal problems such as nausea and or cramping, grade 3 = any of the former grades plus vomiting/diarrhoea and respiratory tract problems such as throat pruritus or tightness, grade 4 = any of the former grades and respiratory arrest plus cardiovascular problems such as hypotension.

IgE immunoreactivity of heated Ara h 1 and Ara h 2/6 was analysed by reverse EAST inhibition. Control experiments were performed using sera from non-allergic subjects and showed no IgE binding at all. Figure 3 shows inhibition of the IgE binding to N-Ara h 1 (Fig. 3A) and N-Ara h 2/6 (Fig. 3B) by N, R+g and R-g allergens obtained for representative sera.

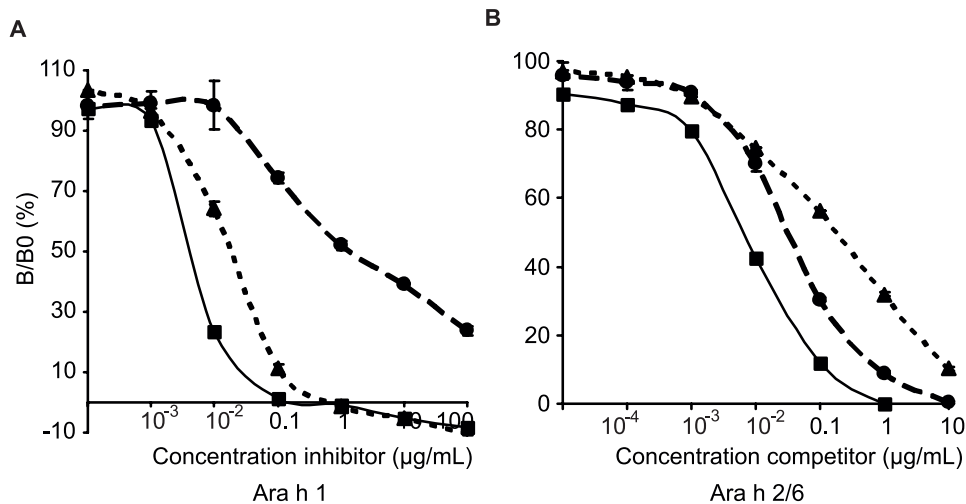


Fig. 3. IgE capture inhibition curves obtained for N, R+g, and R-g Ara h 1 (A) and N, R+g, and R-g Ara h 2/6 (B) as competitors. IgE binding capacities of native and heat modified +/- glucose allergens were assessed by competitive assays in the IgE capture format. Inhibition was performed into microtiter plates coated with anti-IgE, and previously incubated with allergic patient sera at convenient dilution. Competition was conducted accordingly, by adding increasing concentrations of competitors, i.e. native (N; —■—), heated in the presence of glucose (R+g; ---●---) or heated in the absence of glucose (R-g; ---▲---) allergens, at the same time as labelled native allergens (Ara h 1 (A) or Ara h 2/6 (B)). Results are shown of representative sera of peanut-allergic patients (serum #78 for Ara h 1 and serum #70 for Ara h 2/6). Averages are shown of duplicate values \pm SD.

IC50 values obtained for the different sera and allergens were then calculated (Fig. 4). Heating decreased the IgE binding capacity of Ara h 1, IC50 values for R+g and R-g Ara h 1 being \sim 9000- and 3.6-fold higher, respectively, compared with the N-Ara h 1. Heating also decreased the IgE binding capacity of Ara h 2/6, resulting in a 22- and almost 600-fold decrease of the inhibition potency for R+g and R-g Ara h 2/6, respectively, when compared with N-Ara h 2/6.

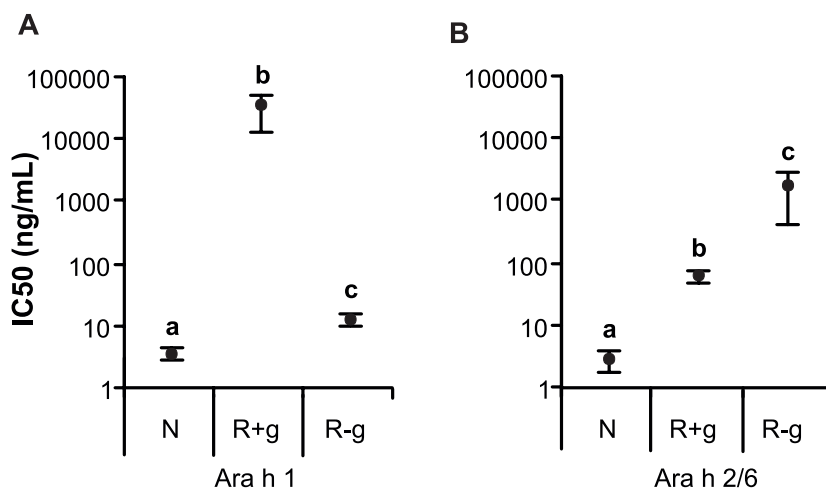


Fig. 4. Analysis of IC₅₀ (ng/mL) values obtained using IgE capture inhibition assay with N, R+g, and R-g Ara h 1 (A) and N, R+g, and R-g Ara h 2/6 (B) as competitors. Protein amounts to obtain 50% inhibition of the Native Ara h 1-tracer (A) or the Native Ara h 2/6 tracer (B) were determined using five (A) or eight (B) individual sera from peanut-allergic patients. The average IC₅₀ values are shown \pm SEM. Increase of IC₅₀ value corresponds to a decrease in IgE-binding capacity. Means without a common letter differ ($P < 0.05$).

The IgE cross-linking capacities of native and heated allergens were initially determined using histamine release tests that were performed employing passively sensitized human stripped basophils. Sera from the selected peanut-allergic patients were first screened to determine the optimal concentration range of the allergens. Sera from two non-allergic controls were included for which histamine release levels were < 10 ng/mL for all allergens (data not shown). Histamine release dose-response curves were similar for all patients' sera tested and titration curves of representative sera are represented in Figure 5. Native Ara h 2/6 (Fig. 5B) demonstrated a profoundly higher biological activity than N-Ara h 1 (Fig. 5A), showing HR of > 10 ng/mL at a protein concentration of $0.001 \mu\text{g/mL}$ compared to the $10 \mu\text{g/mL}$ of N-Ara h 1 needed to elicit equivalent HR. Despite reducing the IgE binding capacity of Ara h 1, heating in the presence or absence of glucose profoundly increased the degranulation potency of this allergen, and showed a HR of > 10 ng/mL at a concentration of $0.01 \mu\text{g/mL}$ for both heated in the presence and absence of glucose (Fig. 5A). In contrast, heating reduced the degranulation activity of Ara h 2/6, particularly in the absence of glucose (Fig. 5B). The different proteins were graded from 1 to 6, a higher grade indicating a higher histamine releasing capacity. Thus, Ara h 1 showed a significantly higher allergen class after heating (independent of the addition of glucose), whilst Ara h 2/6 had a significantly lower allergen class after heating in the absence of glucose.

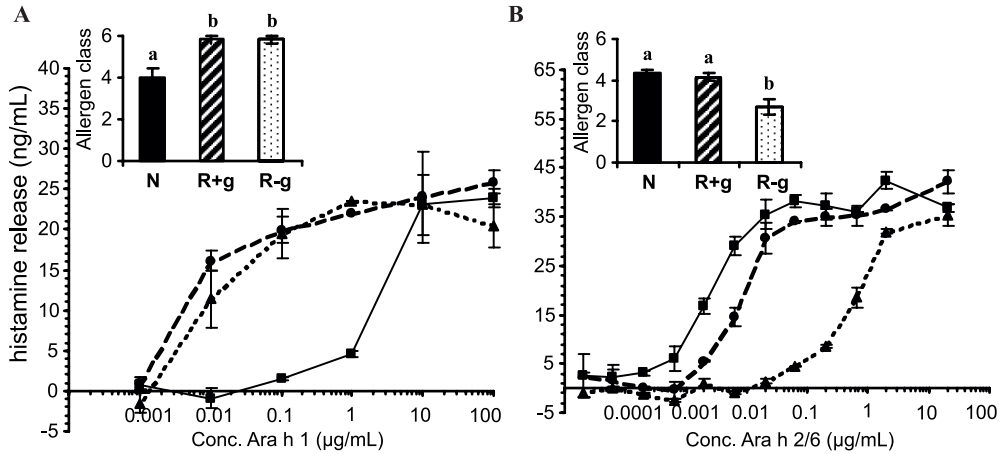


Fig. 5. Histamine release (ng/mL) by passively sensitized basophils induced by (non) modified allergens. Basophils were passively sensitized with sera from a peanut-allergic patient and stimulated with increasing concentrations of native (N; —■—), heated in the presence of glucose (R+g; —●—) or heated in the absence of glucose (R-g; ---▲---) Ara h 1 (A) or Ara h 2/6 (B). Results are shown of representative sera of peanut allergic patients (serum #67 for Ara h 1 and serum #69 for Ara h 2/6). Error bars represent the SD of duplicate values. The column graphs presented within each figure represent the classes of the allergens, based on grading of the responses from 1 to 6. The grading is based on the lowest concentration inducing a histamine release of > 10 ng/mL, in which grade 1 is basophils only reacting to the highest concentration of the allergen tested, and 6 is basophil reaction to the lowest concentration tested ($n = 6-7$). Means without a common letter differ ($P < 0.05$).

The biological mediator-releasing activity of the native *versus* the heat-modified allergens was also assessed in the RBL mediator release assay. The sera that showed significant degranulation capacity in this test were generally those sera having high peanut-specific IgE levels. After passive sensitization of RBL cells with IgE from peanut-allergic patients, the cells were challenged by serial dilutions of the native or processed allergens which resulted in a dose-dependent mediator release. Figure 6 shows typical results of representative sera. In addition, the table presented within each figure represents the average protein concentrations to provoke 50% of the maximum mediator release induced by the native allergen (EC₅₀). The allergenic activity of the thermally-treated Ara h 1 was dramatically increased with a 700- and 1700- fold reduction in EC₅₀ for R+g and R-g Ara h 1, respectively, compared to the native protein (Fig. 6A). The presence of glucose during heating had only a minor influence on the allergenic activity. Similar results were obtained with respect to light scattering, IgE reactivity and RBL degranulation capacity with a different batch of heated and heat-glycated Ara h 1 (data not shown).

In contrast, extensive heating of Ara h 2/6 increased the IC₅₀ values by 19- and 661-fold for R+g and R-g, respectively (Fig. 6B). The presence of glucose significantly moderated this loss in degranulation capacity over heating alone. Non-specific binding to the protein (and possibly other products present after Maillard modification) was not observed as no β -hexosaminidase release was induced by the different allergens when using sera from non-allergic patients (data

not shown). Furthermore, testing the native allergens, N-Ara h 2/6 showed the highest degranulating activity with a maximum release obtained at 1 ng/mL versus 1 µg/mL for N-Ara h 1.

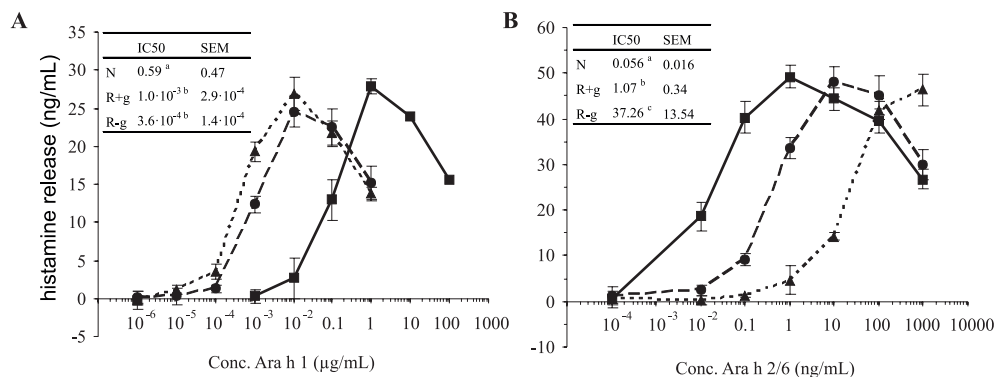


Fig. 6. Percentage of β -hexosaminidase release from humanized rat basophilic leukaemia (RBL) cells induced by (non) modified allergens. Humanized RBL cells were passively sensitized with sera from a peanut-allergic patient and stimulated with increasing concentrations of native (N; —■—), heated in the presence of glucose (R+g; ---●---) or heated in the absence of glucose (R-g; ---▲---) Ara h 1 (A) or Ara h 2/6 (B). Results are shown of representative sera of peanut-allergic patients (serum # 70 for Ara h 1 and serum # 66 for Ara h 2/6). Error bars represent the SD of triplicate values. The table presented within each figure represents the average protein concentrations (μ g/mL for Ara h 1 and ng/mL for Ara h 2/6) to obtain 50% of the maximum allergen release of the native allergen (IC50) ($n = 4-5$). Means without a common letter differ ($P < 0.05$).

Discussion

In order to relate processing effects to the impact on structure of allergens more directly we studied the effects of thermal treatments on the allergenic activity of Ara h 1 and Ara h 2/6 purified from raw peanut. Many thermal treatments employed in the processing of peanuts, such as roasting and frying, are undertaken when the water content is low, usually around 4%. The present paper describes a model treatment for purified allergens which attempts to mimic these processing conditions, using freeze-dried allergens heated at 145°C for 20 min in the presence or absence of glucose. The presence of water can have a profound effect on the denaturation behaviour of proteins as these tend to be more thermostable when water content is limiting [41]. In the present study, the effect of the heating was pyrolysis of the proteins, resulting in their degradation to much smaller polypeptides. However, we observed that these degradation products were assembled into larger structures, mainly for Ara h 1 that is present as very large macromolecular aggregates after extensive heating with glucose. In addition, besides pyrolysis followed by aggregation, also aggregation of monomers resulting in large fragments not dissolvable in SDS might have occurred. The greater thermal stability of the 2S albumins resulted in a greater proportion of the Ara h 2/6 being soluble after application of heat in the dry state compared to Ara h 1, irrespective of whether glucose was included. It seems thus likely that the peptides resulting from pyrolysis will remain assembled into larger

structures. In addition, covalent cross-linking of the protein as a consequence of Maillard reactions [17,42] explains the low protein extraction yield of Ara h 1 heated in the presence of glucose. The soluble fractions used in the current study represent parts of the proteins which might be readily soluble from roasted peanuts and hence involved in the rapid reactions observed in peanut-allergic individuals, particularly in the oral cavity. These were observed to be present in an aggregated form but comprised thermally-induced hydrolysis products, since almost no intact Ara h 1 or Ara h 2/6 remained after the thermal treatment.

The decrease in IgE binding capacity of the thermally treated peanut allergens indicates structural changes induced by heating which results in epitopes having either reduced affinity and/or accessibility for specific IgE. This was particularly marked for Ara h 1 heated in the presence of glucose, which on the basis of the loss of primary amino groups suggests that non-enzymatic glycosylation of the protein changed or destroyed the epitopes. Alternatively, shielding of the epitopes by covalently attached polysaccharide chains or by the higher extent of aggregation may render it less able to bind IgE. In addition, the hydrophilic glucose could have changed the hydrophobicity of the protein resulting in a decrease in IgE binding as the hydrophobic amino acid residues located in the centre of the epitope appeared to be the most critical for IgE binding [10].

Besides determining the IgE binding capacity of the allergen, we also assessed the biological activity of the native and processed allergens, i.e. their potency to cross-link cell bound IgE and to induce effector cell degranulation. Even though a highly significant decrease in IgE binding capacity was observed for heated Ara h 1, its capacity to elicit mediator release was significantly enhanced. Theoretically, more than one IgE binding site is required per fragment of an allergen to induce IgE cross-linking. The R+g Ara h 1 aggregates present a large surface containing multiple copies (of the same) IgE epitopes which clearly enhances the cross-linking capacity of the protein, in comparison to its intrinsic IgE-binding capacity, as defined by immunoassays. There is other evidence that aggregated structures are more effective at triggering histamine release than soluble monomeric forms of the same protein [43]. As both R+g Ara h 1 as well as R-g Ara h 1 had a strongly increased degranulation capacity, we can conclude that these aggregated Ara h 1 structures having a hydrodynamic diameter of at least 30 nm are better in degranulating cells than Ara h 1 being in its native trimeric state having a diameter of 16 nm.

In contrast to Ara h 1, Ara h 2/6 does not form large aggregates, but does form dimers and tetramers following heat-induced denaturation [27,44]. **Although most studies that investigated the effect of processing on allergenicity assessed only the IgE binding capacity, Lehmann *et al.* [45] also studied IgE cross-linking capacity. They observed that even though IgE antibody-binding capacity of peanut 2S albumins was decreased after protease treatment, this did not result in a reduction of the allergenic potential using RBL cells. In our study, heating at 145°C for 20 min decreased IgE binding as well as IgE cross-linking capacity of Ara h 2/6, and the presence of glucose during roasting moderated these losses. This protective effect of glucose was also observed both for the IgE binding and the biological activity for the non-specific lipid transfer protein from apple (Mal d 3) after heating at 100°C for 2 h [23]. Furthermore, we showed that heating had a different effect on Ara h 1 compared with Ara h 2/6 with respect to cross-linking capacity. Heating of Ara h 1 resulted in an increased cross-linking and degranulation capacity, while this was decreased after heating for Ara h 2/6. These data demonstrate that only studying the effect of food processing using IgE binding capacity tests is insufficient to fully assess**

the degree of allergenicity of the (modified) protein and that effects on the biological activity, such as mediator release, must also be considered.

In summary, the effect of roasting on allergenicity in the presence or absence of glucose depends on the allergenic protein tested and cannot be generalised for all proteins. We observed that in both cellular mediator release assays used in this study, heating at low moisture of purified Ara h 1 in the presence or absence of glucose resulted in an increased degranulation capacity, while for Ara h 2/6 the degranulation capacity was decreased. In addition, in contrast to Ara h 2/6, the IgE binding capacity of Ara h 1 did not correlate to IgE cross-linking capacity and conclusions drawn from individual assays have to be taken with care. Gaining more knowledge on the effect of the food matrix and the role of food processing on individual allergenic proteins enables better understanding of their clinical impact, e.g. in terms of severity of reactions. It is also of importance in diagnostic procedures, in particular when food challenges are part of the diagnostic process. Threshold studies of peanut exposure may require standardization of the peanut roasting conditions to make the challenges used in clinical trials comparable. In addition, the results from this study can have important ramifications for approaches using purified components for component resolved diagnosis.

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Chapter 5

The impact of Maillard reaction on immunoreactivity and allergenicity of the hazelnut allergen Cor a 11

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Submitted paper

Abstract

Few studies exist on the influence of processing methods on structural changes and allergenic potential of hazelnut proteins. Our study focused on the effect of glycation (Maillard reaction) on the immunoreactivity and degranulation capacity of the purified hazelnut 7S globulin, Cor a 11. After heating, the extent of the Maillard reaction, sensitivity to proteolysis, binding of human IgE or rabbit IgG, and degranulation capacity were analyzed. Changes in electrophoretic mobility, amount of free amino groups, content of bound sugar and fructosamine indicated that glycation of Cor a 11 occurred at all conditions. Glycation at 37°C did not influence the specific IgG or IgE binding, and was decreased after heating at 60°C and 145°C. Heating, with or without glucose at 145°C increased basophil degranulation capacity. The results suggest that glycation of Cor a 11 at 60°C and 145°C may decrease the IgE/IgG binding properties but not the degranulation capacity of basophils. This is possibly related to aggregation of the proteins as a result of the Maillard reaction.

Introduction

Food allergy is one of the major health concerns in the highly industrialized societies [1,2] with hazelnuts (*Corylus avellana*) being one of the commonest food sensitizers, in terms of presence of IgE in the sera, in Europe [3,4]. Recent research showed that when all birch allergic subjects were excluded from the tested population, hazelnut remained among the four most common food sensitizers, indicating that cross-reactions between the birch pollen allergens Bet v 1 and Bet v 2 and the hazelnut allergens Cor a 1 and Cor a 2, respectively does not explain this high sensitization rate for hazelnut [3]. Non-pollen related hazelnut allergens that have been identified so far are the 7S globulin Cor a 11 [5,6], 11S globulin Cor a 9, lipid transfer protein Cor a 8, hazelnut oleosin and the recently identified 2S albumins from hazelnut [7].

Besides Cor a 11, several 7S vicilin-like seed storage proteins have been identified as important food allergens, including Ara h 1 in peanut, Jug r 2 in walnut and Ana o 1 in cashew [8]. The high homology between different 7S globulins from peanuts and tree nuts leads to the widely observed IgE-binding cross-reactivity [8], and therefore many patients allergic to peanuts have a reaction to other tree nuts and vice versa [9,10]. Hazelnut vicilin Cor a 11 is responsible for oral sensitization through ingestion of the food, and the percentage of positive IgE responses to Cor a 11 in hazelnut allergic patients has been shown to be between 47-95% [5,6]. Molecular properties of Cor a 11 such as protein structure, amino acid sequence, glycosylation sites and epitope localization are described [5,11]. However, data on the influence of food processing conditions on immunoreactivity and allergenic properties of Cor a 11 are scarce, while hazelnuts are used in many processed foods [12]. Moreover hazelnuts have been classified as one of the most common hidden allergens in foodstuffs, especially in sweets [13].

Different processing conditions applied during food manufacturing may alter protein structure and may cause a change in the allergenicity of the whole product [14]. The IgE-binding capacity of hazelnut allergens was shown to be stable to heat treatment [15]. On the other hand Hansen *et al.* showed a decreased allergenicity, in a DBPCFC, of roasted compared to raw

hazelnut [16]. Muller *et al.* identified Cor a 11 as the heat stable allergen which was recognized after roasting (40 min, 145°C) by specific IgE [17]. Reported data suggest that hazelnut allergens possess different susceptibility to heat and it is not clear whether heat treatment may result in a change of the allergenic potential. Additionally, during food processing proteins are mostly treated in the presence of different types of sugar what presents conditions that allow further protein modifications [18,19]. One of the most widespread modifications of proteins that occurs during food processing is the Maillard reaction (glycation) between reducing sugars and amino groups of proteins [20]. A cascade of chemical rearrangements including condensation, oxidation, hydration (and dehydration), leads to formation of numerous Maillard reaction products (MRPs) including components that may impact on human health, such as acrylamide, heterocyclic amines (HCAs) and glycation/lipoxidation end products (AGE/ALEs). This changes bioavailability and nutritional values of food [21] and influences food properties such as flavor, color and taste [22,23]. Recent studies have shown that the Maillard reaction also alters the allergenicity and immunoreactivity of food proteins e.g. by the formation of new IgE binding epitopes or by destruction of existing ones [18,24].

The aim of this study was to determine the influence of the Maillard reaction occurring at three different temperatures on the physicochemical properties, antibody binding and degranulation capacity of the hazelnut allergen Cor a 11. The three different temperatures chosen were: 37°C, which corresponds with the human body temperature and has only a minor effect on protein structure [25], 60°C, which changes the secondary and tertiary structure of proteins [19] and is often applied during food manufacturing [26] and 145°C which is the routine hazelnut roasting condition [27].

Material and Methods

Protein Extraction and Purification

Fresh hazelnuts (*Corylus spp*) from Turkey were ground and defatted with n-hexane (2 times for 15 minutes). Defatted hazelnut flour was extracted according to Rigby *et al.* [11]. The supernatant was collected and proteins were precipitated with ammonium sulphate to 60% saturation. After centrifugation (10000×g, 30 min, 4°C), the supernatant was dialyzed overnight against 50 mM Tris-HCl buffer. Further purification was performed by concanavalin A affinity chromatography (Sigma Aldrich, Germany) [11] and elution was carried out with 50 mM α-D-methylmannoside (Sigma Aldrich, Germany). Protein containing peak fractions were pooled and dialyzed overnight against phosphate buffered saline (PBS), pH 7.4 at 4°C. The protein concentration was assessed by the BCA-assay (Pierce, USA) and purity was determined by SDS-PAGE as described below.

Protein Glycation

The protein solution was split into seven batches of 10 mg each and contained 0.01% sodium azide. To three batches, glucose (Sigma Aldrich, Germany) was added in a ratio of 1:2 (w/w protein:glucose) [28]. All seven samples were placed into glass bottles, frozen at -70°C and lyophilized (Christ, Alpha 1-4 LSC). Next, the lyophilized protein samples (one with glucose and one without) were heated for 7 days at 37°C or for 3 days at 60°C in a laboratory incuba-

tor with gravitational circulation of air (Wamed, C-30G), or for 20 minutes at 145°C in a forced convection chamber of thermal research (Wamed, KBC 65W). One batch was not treated and regarded as the native, non-modified form. After treatment, the samples were allowed to gradually cool to room temperature. Proteins were slowly dissolved in Milli-Q water, centrifuged (5000×g, 15 min, 4°C) and supernatant was collected and ultrafiltrated (Millipore, Amicon Ultra-15, 10,000 NMWL) against PBS for several rounds to remove the residual glucose and other small molecular weight products. The protein concentration and changes in solubility were assessed by the BCA-assay (Pierce, USA) and aliquots were stored at -70°C. All treatments were performed in duplicate.

SDS-PAGE Analysis

Native (non-modified) and modified Cor a 11 samples were separated by SDS-PAGE under reducing conditions. Protein samples in SDS-sample buffer were boiled and loaded (10 µg/lane) on a 12% polyacrylamide gel and afterwards stained using colloidal Coomassie (Sigma Aldrich, Germany) [29]. A molecular weight marker ranging from 6.5 to 200 kDa (Sigma Aldrich, Germany) was included and molecular masses were calculated by the use of GelScan 1.10 software.

o-Phthaldialdehyde (OPA) Assay

The OPA assay was applied to determine the proportion of reacted amino groups of Cor a 11 in glycosylated and control samples. Protein samples (250 µg/ml) were mixed with the OPA reagent [30] in a ratio of 1:10 (v/v). The mixed solutions were incubated for 20 minutes at room temperature and the absorbance was measured at 340 nm against a control containing PBS and the OPA reagent. Unreacted amino groups were estimated from a calibration curve established with L-leucine.

Anthrone-Sulfuric Acid Colorimetric Microassay

The anthrone-sulfuric acid colorimetric microassay was performed to analyze bound glucose. The reagent was freshly prepared by dissolving 20 mg anthrone in 10 ml of 14 M sulphuric acid. The protein solutions (250 µg/ml) were mixed in 96-well plates (Sigma Aldrich, Germany) with the cooled reagent in a ratio of 1:10 (v/v) [31]. A glucose standard curve was used to calculate the sugar content.

Nitroblue Tetrazolium (NBT) Assay

The NBT method is based on the ability of ketoamines (fructosamines) to act as reducing agents in alkaline solutions. The reagent was prepared by mixing solution A (0,82 mM nitroblue tetrazolium, 7 mM sodium cholate, 70 mM KCl in 70 mM phosphate buffer, pH 8.2 including 3% of Tween-20) and solution B (833 mM potassium carbonate buffer, pH 11.2) in a 14:6 ratio. Subsequently, mixture and sample were mixed (19:1 ratio) and incubated at 37°C. Fructosamine (320 µM/L) was used as a standard and absorbance was measured in a microplate reader (Asys UVM 340) at 550 nm after 9 and 10 minutes of incubation. The results were calculated using the following formula:

$$\text{fructosamine concentration } [\mu\text{mol}] = [(\Delta A \text{ sample} - \Delta A \text{ blind sample}) / (\Delta A \text{ calibrator} - \Delta A \text{ blind sample})] \times \text{calibrator concentration}$$

Protein Hydrolysis

For pepsin hydrolysis, the protein solution was adjusted to pH 2.2 using 1 M HCl. Pepsin (3,200-4,500 units/mg protein, Sigma Aldrich, Germany) was dissolved in simulated gastric fluid (0.15 M NaCl, adjusted to pH 2.0 with HCl) and added to the protein solution in a final enzyme-substrate ratio of 1:10 (w/w). The hydrolysis was performed for 1 h at 37°C with agitation. The enzyme was inactivated by increasing the pH of the samples to 7 - 8 with 1 M NaOH and subsequently samples were directly cooled in an ice bath. The degree of hydrolysis was determined by measuring the increase in the number of free α -amino groups after the hydrolysis using the OPA method as described before [26].

Production of Antibodies

Antibodies were produced using three New Zealand white rabbits by mixing 1 mg of native purified Cor a 11 to 1 ml of sterile PBS emulsified with an equal volume of Freund's adjuvant (Sigma Aldrich, Germany). Complete Freund's adjuvant was used in the first dose and an incomplete Freund's adjuvant in the following doses. The rabbits were immunized subcutaneously, three times, at three-week intervals. The blood samples obtained from a marginal rabbit ear vein were collected every three weeks and the increase of IgG titer was monitored by performing an indirect ELISA. Ten days after the last immunization, the rabbits were exsanguinated. The blood was incubated for 1 h at 37°C and centrifuged at 1500 \times g for 20 minutes. Serum IgG antibodies were purified using ammonium sulphate precipitation (20% saturation). After centrifugation at 1500 \times g for 30 min, the pellet was dissolved in a phosphate buffer of pH 7.4 and dialyzed for 15 h at 4°C. The IgG fraction was lyophilized before use and purity was estimated by SDS-PAGE. The Local Care Use of Animals Committee approved animal handling and experimental procedures.

Competitive ELISA

Competitive ELISA was used to assess immunoreactivity as the ability of a polyclonal anti Cor a 11 antiserum to recognize Cor a 11 after the different treatments. All ELISA steps were performed at 37°C in a thermo-shaker (ELMI). Microtiter 96-well plates (Corning, medium binding) were coated with native Cor a 11 (100 ng/well) in a sodium carbonate buffer pH 9.6. Plates were incubated for 1 h and residual free binding sites were blocked with 1% BSA (Sigma Aldrich, Germany) in PBS for 30 minutes. Then, plates were washed three times with PBS containing 0.5% Tween-20. Native Cor a 11 or the competing antigen (modified Cor a 11) in PBS was added (50 μ l/well) in increasing concentrations ranging from 2×10^{-3} to 2×10^4 ng/ml. Immediately afterwards, rabbit polyclonal anti-Cor a 11 antibodies (5 μ g/ml in PBS, 50 μ l/well) were added. The plates were incubated for 1 h and then washed four times. Peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma Aldrich, Germany) were added (100 μ l/well). The plates were incubated for 1 h and washed four times. The substrate, OPD (Sigma Aldrich, Germany) in 50 mM citrate-phosphate buffer, pH 5.0 was added in a ratio of 1:2 (w/v) with 0,6% hydrogen peroxide (100 μ l/well). After 30 minutes of incubation the enzymatic reaction was stopped by addition of 5 M HCl (50 μ l/well) and the absorbance was measured at 492 nm on an automatic plate reader (Asys UVM 340). The analysis was performed four times in triplicate each time. A maximal signal (100%) was obtained by not adding a competitor and a negative control by omitting the Cor a 11 antibodies. Results were expressed as B/B_0 , where B is the mean ab-

sorbance of the known concentration of inhibitor and BO represent the maximal absorbance obtained in the absence of inhibitor. Using sigmoid curves, the EC_{50} values were read and percentages of cross-reaction were calculated using the following formula:

$$CR\% = (EC_{50}N / EC_{50}C) \times 100\%$$

where CR% - percentage of cross-reaction, $EC_{50}N$ - concentration of specific antigen needed to bind 50% of the available antibodies, $EC_{50}C$ - concentration of cross-reacting antigen needed to bind 50% of the available antibodies.

Dot-Blot Immunoassay

To test the specific IgE binding capacity of Cor a 11 after the different treatments, an immunodot blot assay was performed using pooled sera obtained from eight hazelnut allergic patients with specific IgE against hazelnut varying from 0,9 to 33,4 kU/l. The diagnosis of hazelnut allergy was based on clinical history and IgE measurement. Double-blind, placebo-controlled food challenges were not performed because of the severities of the systemic reactions. Native and heated Cor a 11 (1.25 and 2.5 μ g) was spotted on the nitrocellulose membrane (Millipore). Nitrocellulose membranes were air-dried and unbound protein-binding sites were blocked using 5% skim milk powder (w/v) and 0.1% Tween 20 (v/v) in PBS. After each step the membranes were washed 4 times with PBS containing 0.1% Tween 20. The allergic sera were diluted 1:3 in PBS with 0.01% BSA and incubated with the membranes for 2.5 h at 37°C with gentle shaking. After washing, the membranes were incubated during 1 h with mouse anti-human IgE (Sigma Aldrich, Germany) diluted 1:1000. The signal of the reaction was developed with 3,3'-Diaminobenzidine (DAB, Sigma Aldrich, Germany) and the membranes were scanned on an ImageScanner (GE Healthcare). The optical density was analyzed by the use of GelScan v1.10 software.

Mediator Release Assay (MRA) using Rat Basophilic Leukemia (RBL) Cells

Sera used in the MRA were derived from healthy ($n = 2$) and peanut allergic ($n = 5$) donors and sera high in Cor a 11 specific IgE ($n = 2$). First, concentrations of IgE specific to 7S globulins purified from hazelnut and peanut were determined using the enzyme allergosorbent assay (EAST) as previously described [32].

RBL-2H3 cells stably expressing the α -chain of the human Fc ϵ -RI receptor [33] were kindly provided by Drs. Vieths and Vogel (Paul-Ehrlich-Institut, Langen, Germany). Cells were cultured in MEM medium supplemented with 5% FCS and 1% glutamine (all from Gibco, Paisley, UK) at 37°C in a humidified atmosphere with 5% CO₂. Cells in stationary growth phase (which were more than confluent) were harvested and plated in 96-well plates at 1.5×10^5 cells/well. Human sera were added at a pre-determined convenient dilution of 1:30 and incubated overnight to passively sensitize the cells. After washing, the cells were stimulated for 1 h with the allergens diluted in Tyrode's buffer containing 50% deuterium oxide [33]. The antigen-specific release was quantified by measuring β -hexosaminidase activity and expressed as percentage of the total β -hexosaminidase content that was obtained by lysing the cells with Triton-X100 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Spontaneous release was determined on cells not sensitized nor cross-linked. The release data were fitted to four-parameter-logistic curves by non-linear regression using the SigmaPlot v10.0 software package, and EC50 values were

assessed accordingly.

Statistics

Results were expressed as mean \pm SD of three to five independent measurements (each measurement was performed in triplicate). Statistical analysis was carried out by GraphPad Prism 4 software. A one-way ANOVA test with Tukey post-hoc ($P < 0.05$) was used to evaluate the significance.

Results

The Influence of Thermal Processing in the Presence and Absence of Glucose on Physicochemical Properties of Cor a 11

The temperature-dependent changes in Cor a 11 solubility and color after heat treatments are shown in Table 1. Solubility of protein decreased with increasing temperature and with the presence of glucose during the heating at 60°C and 145°C. Heating at 37°C reduced the solubility only slightly, while heating at 60°C decreased the solubility of Cor a 11 to 91% after heating without the presence of glucose and 65% after heating in the presence of glucose. Around 76% and 9% of Cor a 11 remained soluble after heating at 145°C in the absence and presence of glucose during heating, respectively.

Table 1. The influence of heat treatment on Cor a 11 solubility and color changes.

	C37°C	G37°C	C60°C	G60°C	C145°C	G145°C
Solubility (%)	97 \pm 2.8	95 \pm 2.5	91 \pm 4.9	65 \pm 1.4	76 \pm 10.5	9 \pm 1.7
Colour	transparent	transparent	transparent	light brown	light brown	brown

Average percentage of solubility of two heating-treatments performed independently \pm SD

SDS-PAGE analysis under reducing conditions (Figure 1) showed a purity of the Cor a 11 of 98% (lane 1) of which ~90% consisted of the main subunit with a molecular mass estimated to be 47 kDa. For all treatments, differences in the SDS-protein pattern were observed between Cor a 11 heated in the absence and presence of glucose. 38% and 52% of the total fraction of Cor a 11 heated at 37°C in the absence and presence of glucose respectively, degraded (line 2 and 3), and was observed as two or three bands. Furthermore, in the sample treated without glucose an additional band with a molecular weight higher than 200 kDa was observed, which constituted about 9% of the total fraction. Cor a 11 heated at 60°C in the absence of glucose partly degraded into two low molecular weight proteins (line 4), while the protein heated in the presence of glucose appeared to be more stable and was seen as one band (line 5) with a molecular weight slightly higher than native Cor a 11 (line 1). Heating at 145°C caused aggregation of Cor a 11; however, 40% of Cor a 11 heated at 145°C without glucose appeared as a 47 kDa band (line 6), similar to the native Cor a 11. Cor a 11 heated at 145°C in the presence of glucose resulted in a total loss of the 47 kDa protein and proteins appeared as a smear in the range of molecular masses higher than the mass of the native protein (line 7).

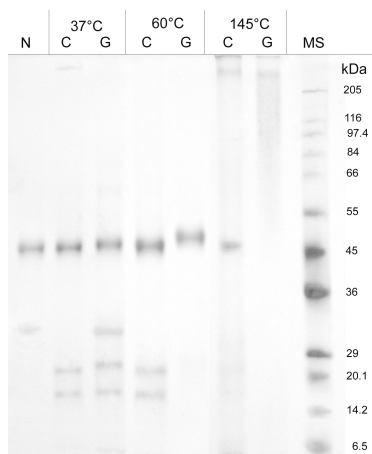


Figure 1. SDS-PAGE patterns of native and treated Cor a 11. N – native (non-modified) protein; **C37°C** – control sample (without glucose) treated at 37°C for 7 days; **G37°C** – sample treated in the presence of glucose at 37°C for 7 days; **C60°C** – control sample (without glucose) treated at 60°C for 3 days; **G60°C** – sample treated in the presence of glucose at 60°C for 3 days; **C145°C** – control sample (without glucose) treated at 145°C for 20 minutes; **G145°C** – sample treated in the presence of glucose at 145°C for 20 minutes; **MS** – molecular weight marker [kDa]. Arrow indicates the molecular mass corresponding with the mass of native form of Cor a 11.

To assess the extent of the Maillard reaction after heating at these different temperatures, three analytical methods have been applied. The OPA analysis (Figure 2) showed a not significant 10% loss of primary amino groups in the protein heated at 37°C in relation to the native protein. A 50% and 58% decrease of free amino groups was observed for Cor a 11 heated at 60°C and 145°C, respectively, which suggests an increase in the rate of the reaction of glucose with the ϵ -amino group of lysine or the α -amino groups of terminal amino acids at higher temperatures. When analyzing the subsequent temperatures of modification, the amount of free amino groups was negatively correlated with the amount of protein-bound sugar (Pearson $r = -0.856$, $P = 0.029$; Figure 2). No differences in the percentages of primary amino groups and bound sugar were observed between all samples heated without the presence of glucose and the native protein (data not shown). An increase in fructosamine content was observed in all protein samples heated in the presence of glucose for all tested temperature conditions (Figure 3). This indicates that the reaction has reached at least the second stage of the Maillard reaction, the formation of Amadori products. The brown color of Cor a 11 glycosylated at 60°C and 145°C is most likely a result of involvement of fructosamine which leads to the formation of insoluble brown products (Table 1) [23].

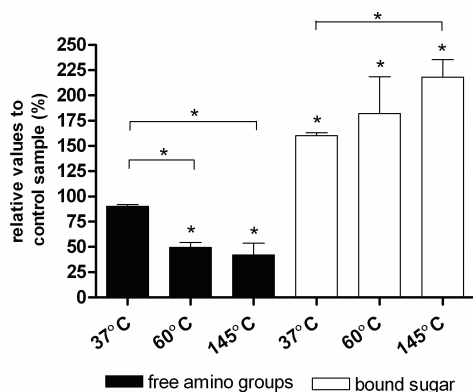


Figure 2. Percentage of primary amino groups (black bars) and bound sugar (white bars) in relation to native Cor a 11 (100%). Data are reported as the mean \pm SD ($n = 5$ for free amino groups, $n = 4$ for bound sugar). * $P < 0.05$ compared to native Cor a 11 (lower part); statistical differences between Cor a 11 treated at different temperatures (upper part).

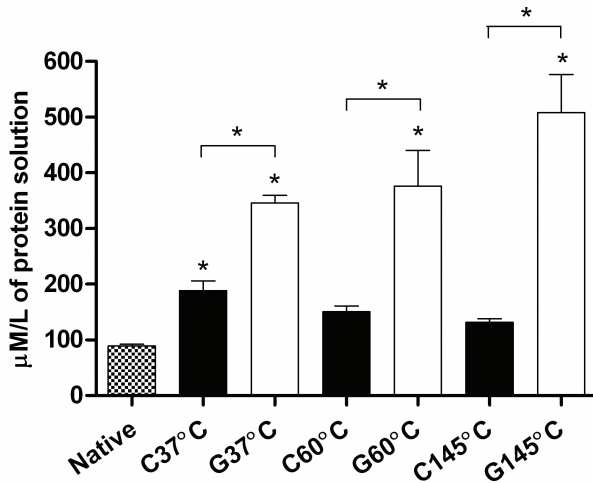


Figure 3. The level of browning products determined with the NBT assay of Cor a 11 treated without glucose – control (C) and in the presence of glucose – glycation (G). Data are reported as the mean \pm SD. Analysis was performed in fivefold and repeated once for the first batch and two times for the second batch ($n = 5$). * $P < 0.05$ compared to native Cor a 11 (lower part); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper part).

The high degree of Maillard reaction observed after heating at 60°C and 145°C in the presence of glucose (Table 1, Figure 2 and 3) was accompanied with a significant decrease in susceptibility of Cor a 11 to pepsin hydrolysis. The degree of Cor a 11 pepsin hydrolysis (DH) is presented as a percentage of cleaved peptide bonds (Figure 4). Significant differences were observed in the susceptibility to hydrolysis between Cor a 11 heated in the presence and absence of glucose. A 5% increase of the susceptibility to hydrolysis was observed after heating at 37°C in the absence of glucose, while heating in the presence of glucose showed the same DH as native Cor a 11. The structural changes of Cor a 11 after heating at 60°C in the absence of glucose, resulted in a 60% increase of susceptibility to hydrolysis in relation to the native protein. After glycation at 60°C, a 95% decrease of hydrolysis was observed, while heating at 145°C decreased the degree of hydrolysis to 30% in the absence and 62% in the presence of glucose.

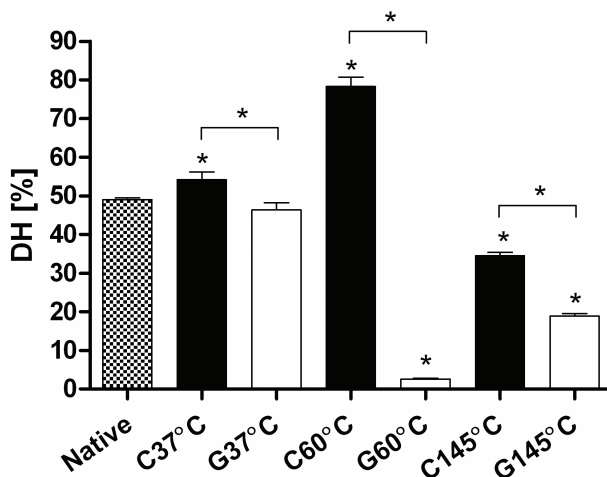


Figure 4. Hydrolysis degree of Cor a 11 treated without glucose – control (C) and in the presence of glucose – glycation (G). Data are reported as the mean \pm SD ($n = 3$). * $P < 0.05$ compared to native Cor a 11 (lower part); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper part).

The Influence of Thermal Processing in the Presence and Absence of Glucose on Antibody Binding Properties

The immunoreactivity was determined quantitatively as the percentage of cross-reactivity between native and modified Cor a 11 with the use of polyclonal rabbit IgG antibodies against native Cor a 11 (Figure 5). Glycation of Cor a 11 at 37°C did not alter its immunoreactivity significantly, while heating at 37°C without sugar resulted in a 30% decrease in immunoreactivity as reflected in Figures 5A and B. Heating of Cor a 11 in the presence of glucose at 60°C triggered loss of immunoreactivity of 53% while in the non-glucose control heating to 60°C resulted in an increase of the immunoreactivity of Cor a 11 of 28% (Figure 5A, C). The extent of cross-reactivity between native Cor a 11 and Cor a 11 heated at 145°C was estimated to be 2% and no cross-reactivity was observed between native Cor a 11 and Cor a 11 glycated at 145°C (Figure 5A, D).

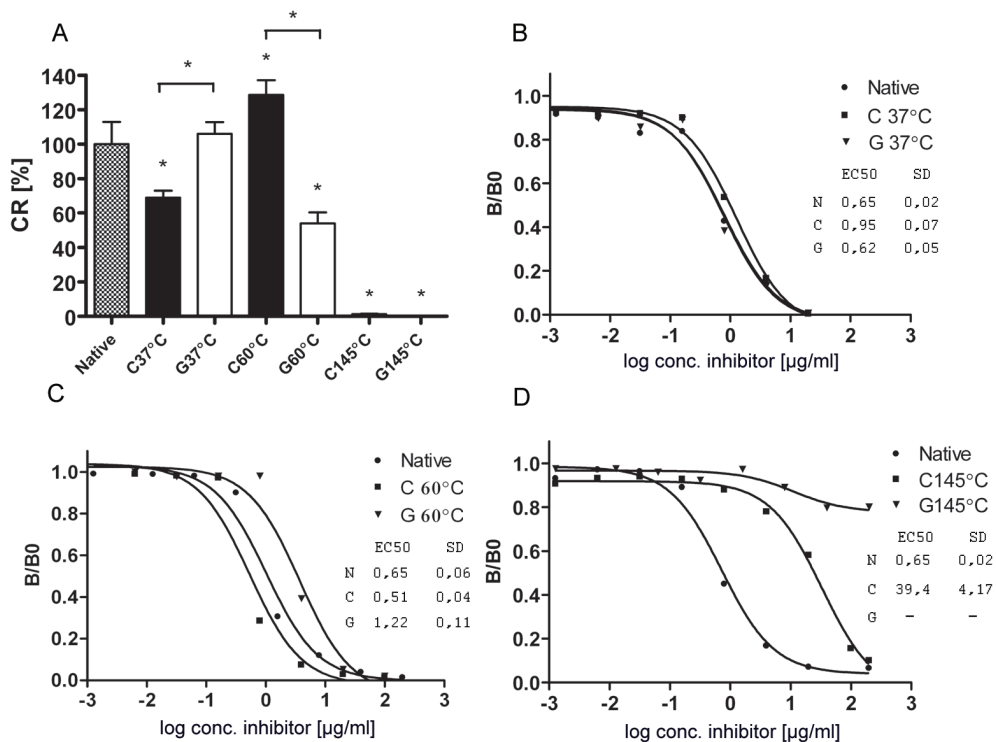


Figure 5. Competitive ELISA inhibition of the binding between treated Cor a 11 and its specific polyclonal anti-native Cor a 11 antibodies. The plates were coated with native Cor a 11. **A.** The results expressed as the percentage of cross-reactivity in relation to native Cor a 11 (100%). Cor a 11 treated without glucose - control (C), Cor a 11 treated in the presence of glucose - glycation (G). **B-D.** Representative curves obtained for Cor a 11 treated at 37°C, 60°C and 145°C, respectively. Data are reported as the mean \pm SD ($n = 4$). * $P < 0,05$ compared to native Cor a 11 (lower part); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper part). The table presented within each figure represents the average protein concentrations ($\mu\text{g/ml}$) to obtain 50% of the maximum allergen release of the native allergen (EC50) ($n = 5-8$).

The binding properties of the native and heated Cor a 11 to allergen-specific IgE antibodies were analyzed in immuno-dot assays performed with the use of pooled sera from 8 hazelnut allergic patients (Figure 6). The specificity of the reaction was tested using pooled sera from 10 non-allergic patients (data not shown). Crude hazelnut extract, purified Ara h 1 (7S globulin isolated from peanut), and ovalbumin and ovomucoid as common food allergens were used as control proteins. The highest IgE binding reactivity was shown for the crude hazelnut extract, resulting in intensive dots with a high optical density. No signal was observed for ovalbumin and ovomucoid; however, a positive reaction for Ara h 1 was observed (data not shown). No significant differences in IgE reactivity between native Cor a 11 and the proteins modified at 37°C was observed. A decreased capacity to bind to Cor a 11 was observed after heating to 60°C in the presence of glucose. A significant reduction in IgE reactivity was observed after treatment at 145°C without sugar. The IgE antibodies from the serum pool did not recognize Cor a 11 treated at 145°C in the presence of glucose in the tested concentrations (Figure 6).

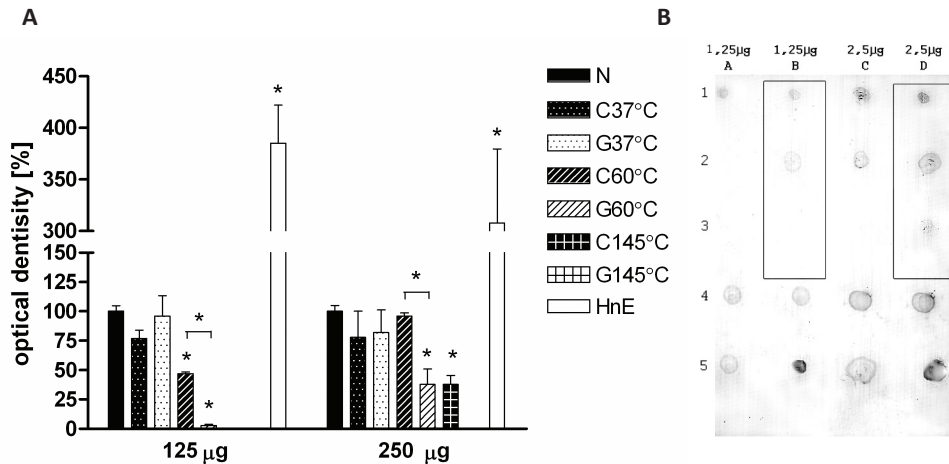


Figure 6 A. Reactivity of human IgE in immuno-dot assay calculated as the percentage of optical density in the relation to the native Cor a 11 (100%). **N** – native (non-modified) protein; **C37°C** – control sample (without glucose) treated at 37°C for 7 days; **G37°C** – sample treated in the presence of glucose at 37°C for 7 days; **C60°C** – control sample (without glucose) treated at 60°C for 3 days; **G60°C** – sample treated in the presence of glucose at 60°C for 3 days; **C145°C** – control sample (without glucose) treated at 145°C for 20 min; **G145°C** – sample treated in the presence of glucose at 145°C for 20 min; **HnE** – the pure extract of hazelnut proteins; Data are reported as the mean \pm SD ($n = 3$). * $P < 0,05$ compared to native Cor a 11 (lower part); statistical differences between Cor a 11 treated in the presence and absence of glucose (upper part). **B.** Human IgE immuno-dot assay. The first two rows show Cor a 11 dotted on a PVDF membrane in the amount of 1.25 µg per dot in the following order: protein treated in the presence (row A) and absence (row B) of glucose. The second two rows present Cor a 11 dotted in the amount of 2.5 µg per dot in the same order: protein treated in the presence (row C) and absence (row D) of glucose. Numbers from 1 to 3 indicate treatments: 1 – Cor a 11 treated at 37°C for 7 days; 2 – Cor a 11 treated at 60°C for 3 days; 3 – Cor a 11 treated at 145°C for 20 minutes; 4 – the native (untreated) Cor a 11 in both concentrations in duplicate; 5B and 5D – a hazelnut crude extract; 5A and 5C – peanut 7S globulin Ara h 1, 6A and 6C – ovalbumin; 6B and 6D ovomucoid.

The Influence of Thermal Processing in the Presence and Absence of Glucose on Mediator Release Assay using RBL Cells

The biological mediator-releasing activity of the native versus the 145°C heated Cor a 11 was assessed in the RBL MRA. First, concentrations of IgE specific to 7S globulins purified from hazelnut and peanut were determined for the included sera. The level of Cor a 11 specific IgE was overall very low for the sera from peanut allergic patients (1-12 kU/l) and did not correlate with the specific IgE to total hazelnut extract; however, it did correlate with peanut 7S specific IgE (data not shown). In addition, IgE levels of two sera containing high Cor a 11 specific IgE were 57.8 kU/l and 47.8 kU/l and both sera contained >100 kU/l of hazelnut specific IgE.

No β -hexosaminidase release was induced by the native and heated Cor a 11 allergens when sera were used from patients not sensitized to hazelnut (data not shown). Figure 7 shows typical results of two representative sera. In addition, the table presented within each figure represents the average protein concentrations to obtain 50% of the maximum mediator release induced by the native allergen (EC₅₀). When sera from five peanut allergic patients were used (Figure 7A), no significant difference in degranulating capacity was observed between native and 145°C heated Cor a 11 (EC₅₀ for all sera and all proteins between 0.6 and 6.8 μ g/ml). However, EC₅₀ values for Cor a 11 heated at 145°C in the presence or absence of glucose were respectively ca. 100- and 300-fold lower than those for native purified Cor a 11 when using the two sera high in hazelnut 7S-specific IgE. Furthermore, for native Cor a 11, the maximum release was obtained between 10-100 μ g/ml using the peanut allergic sera, while this maximum release was already observed at \sim 1 μ g/ml using the two sera high in hazelnut 7S specific IgE.

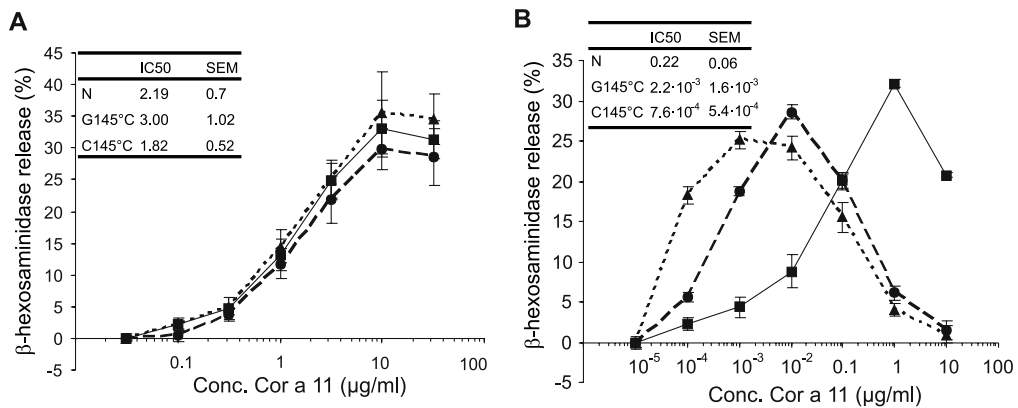


Figure 7. Percentage β -hexosaminidase release from humanized rat basophilic leukaemia (RBL) cells induced by native and modified Cor a 11. Humanized RBL cells were passively sensitized with either sera from peanut allergic patients ($n = 5$) or sera high in Cor a 11 specific IgE ($n = 2$) and stimulated with increasing concentrations of native (N; $\text{---}\blacksquare\text{---}$), heated in the presence of glucose (C145°C; $\text{---}\bullet\text{---}$) or heated in the absence of glucose (C145°C; $\text{---}\blacktriangle\text{---}$) Cor a 11. Results are shown of one representative serum of a peanut allergic patient (A) and a sera high in Cor a 11 specific IgE (B). Error bars represent the SD of triplicate values. The table presented within each figures represents the average protein concentrations (μ g/ml) to obtain 50% of the maximum allergen release of the native allergen (EC₅₀) ($n = 2-5$).

Discussion

Food manufacturing uses thermal processing to improve food quality, safety and shelf life [34]. However, food processing results in changes of protein structure which may alter allergenic properties [20]. Moreover, processing may result in formation of neoallergens. This may partially explain tolerance to unprocessed food by some allergic patients who have been sensitized against processed food [35,36]. Even though widely-used food antigen tests in commercial laboratories mostly use raw food isolates, by testing a large variety of commercially available food products, it has been shown that 31% of the included allergic individuals showed higher IgE antibodies against processed food antigens as compared to the raw food antigens [37].

The extent of the Maillard reaction and the type of reducing sugar are important parameters that are of importance for the biological properties of food derived proteins [22]. Structural characteristics of a protein (e.g. its primary structure and the accessibility of residues to reaction) are crucial for their propensity to participate in Maillard reactions [23,25,28,38]. In this study, three different temperatures of glycation were applied which resulted in different types of Maillard reaction products, namely early stage Amadori rearrangement product (37°C), more advanced products of their degradation (60°C) and finally advanced glycation end products (145°C) [23]. This enabled us to study the effect of different degrees of glycation on the immunoreactivity and degranulation capacity of Cor a 11. Pedrosa *et al.* showed an effective Maillard reaction of pea vicilin carried out at 37°C for 50 h with glucose, galactose, lactose and galacturonic acid while the structure of the protein was not significantly affected [25]. The lack of formation of colored products from Cor a 11 glycated at 37°C suggests an early stage of Maillard reaction which did not alter the susceptibility of Cor a 11 to pepsin hydrolysis nor the binding properties of rabbit IgG and human IgE to the treated protein. Similar results have been obtained for pea albumins glycated at 37°C during 7 days [38]. These data suggest that the structural changes caused by sugar moieties attached to the protein structure at 37°C or by the increase in temperature did not alter the immunological potential of the protein significantly.

From the SDS-PAGE protein pattern of Cor a 11 heated at 60°C, a protective effect of glucose on heat degradation was observed. A similar effect was observed by Maleki *et al.* who showed that the presence of sugar during heating of Ara h 1 and Ara h 2 purified from peanut at 55°C made the proteins more resistant to degradation [22]. Heating at 55-70°C induces a loss of tertiary and secondary structure of proteins, which makes the Maillard reaction occur more efficiently [23]. The degradation of Amadori compounds mainly through 2,3-enolisation and fission leads to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins [23]. The effect of an advanced Maillard reaction was apparent from the SDS-PAGE pattern of Cor a 11 heated at 145°C in the presence of glucose, as a smear was observed caused by cross-linking and non-cross-linking heterogeneous advanced glycation products [22,39,40]. The brown color of Cor a 11 glycated at 145°C, as well as the characteristic smell, are additional indicators of advanced Maillard reaction which were also observed by other researchers studying the properties of roasted peanuts [40-42].

The structural changes in soluble Cor a 11 upon subsequent steps of Maillard reaction occurring at 60°C and 145°C resulted in decreased hydrolysis by pepsin. The Maillard reaction

was previously shown to decrease the susceptibility of different proteins to pepsin hydrolysis [43,44]. The 30% increase of susceptibility of Cor a 11 to pepsin hydrolysis observed by the effect of heating at 60°C in the absence of glucose may be explained by temperature-induced reduction of disulfide bonds, which is an important parameter in protein digestibility [45] in combination with a destabilization of the globular fold, as observed by Koppelman *et al.* [40]. Moreover, the thermal denaturation of proteins treated at 145°C without glucose also decreased the digestibility of Cor a 11, probably due to protein aggregation [46]. It has to be remembered, that the analysis concerned only 9% of the total Cor a 11 fraction which was soluble after glycation at 145°C. The reduced solubility of roasted proteins was observed also by Kopper *et al.* who observed a loss of soluble protein from peanut flour after 10 minutes of roasting at 178°C [47] and similar results were observed for purified Ara h 1 treated at 145°C (Vissers *et al.*, submitted). Covalent cross-linking of the protein as a consequence of Maillard reactions and formation of aggregates after heating explains the low protein extraction yield of Cor a 11 heated in the presence of glucose.

The physicochemical changes of Cor a 11 heated at 60°C in the presence of glucose caused significantly reduced IgG and IgE binding (2-fold and 3-fold respectively) whereas no reactivity was observed in the case of Cor a 11 heated at 145°C in the presence of glucose. Interestingly, a differential effect was observed for Cor a 11 heated at 60°C in the absence of glucose showing an increase in IgG binding, while the IgE binding did not change or even decreased. The main effect of Maillard reaction on the conformational structure of proteins involves changes in hydrophobic interactions [48]. The observed decrease in IgG/IgE binding may result from changes in hydrophilic/hydrophobic areas and net charge at the protein surface caused by attached sugar moieties. It was shown that masking of hydrophobic amino acid residues located in the center of the epitope was shown to be the most critical for IgE binding to Ara h 1 [49]. The strong reaction of specific IgE from human serum with crude hazelnut extract indicates that the patients were allergic to different hazelnut proteins and some patients were most probably not allergic to Cor a 11.

The decrease of IgE binding after treatment of Cor a 11 at 145°C led us to perform the basophil degranulation tests. An increased mediator-releasing capacity was observed for Cor a 11 roasted both in the absence and presence of glucose, when using sera from the patients with a high titer for Cor a 11 specific IgE antibodies. This corroborates our previous study in which we showed an increase in degranulating capacity of the 7S globulin from peanut after roasting by the use of sera from peanut allergic patients (Vissers *et al.*, submitted). However, roasting had no influence on the cross-linking capacity using sera from peanut allergic patients with low Cor a 11 specific IgE. Lauer *et al.* showed that glycosylated native Cor a 11 induced a similar histamine release from basophils compared to recombinant Cor a 11 [5]; however, the sera used were not tested for the amount of Cor a 11 specific IgE present, and high concentrations of the protein were needed to obtain a histamine release. These results would correspond to our results obtained using our sera low in HN 7S specific IgE. De Leon *et al.* speculated that the affinity of cross-reactive IgE antibodies for cross-reactive allergens might be low and therefore high allergen concentrations may be required to trigger basophils [50]. The relation between IgE-binding potential and clinical symptoms is influenced by physical properties (e.g. stability and size) and immunologic properties (affinity and epitope valence) [51]. Therefore, IgE used in the assays should preferably be obtained from individuals highly allergic to the protein tested,

and it is important to have well-characterized patients. This is even more important as different results were obtained using sera containing most probably only cross-reactive IgE to Cor a 11, and sera showing high IgE titers to Cor a 11. In addition, IgG/IgE binding capacity did not correlate to IgE cross-linking capacity and conclusions drawn from individual assays have to be taken with care.

In conclusion, we showed that the impact of Maillard reaction on the biological and allergenic properties of Cor a 11 strongly depends on the conditions of treatment and are correlated with time and temperature of the reaction. Therefore, the Maillard reaction occurring during processing of hazelnuts may either not influence the physicochemical and biological properties of Cor a 11 significantly (treatment at 37°C), or may completely change its allergenic potential (treatment at 145°C). Moreover in our study we demonstrated that the use of a combination of tests is of importance to estimate the clinical relevance of modification of the allergenicity of Cor a 11.

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Chapter 6

IgE component-resolved allergen profile and clinical symptoms in soy and peanut allergic patients

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Summary statement: Soy IgE or soy CRD are both not sufficient to determine soy allergy.

Component-resolved diagnostics (CRD), evaluating the IgE profile of individual allergens, provides deepened insight into diagnosis of food allergy, as recently described for peanut allergy in children [1]. In soy-allergic patients, the soy seed storage allergens Gly m 5 and Gly m 6 have been associated with severe allergic reactions [2], while the clinical importance of the major soy allergen Gly m 4 is debated [3,4]. **Although individual soy and peanut allergens share sequence homology and serological cross-reactivity, few studies exist on soy 2S albumins even though related 2S albumins from peanut (Ara h 2/6/7) are implicated as clinically important allergens.**

The goal of this study was to determine if CRD could better predict the clinical relevance of specific allergens inducing IgE sensitization to soy, in peanut and/or soy allergic patients.

The medical records of patients seen at an allergy clinic (APA, Arnhem, the Netherlands) over the past 4 years were digitally screened for a clear medical history of anaphylactic reactions to peanut and/or soy. Of these, all adult patients with a positive skin prick test to commercial peanut and/or soy extract (ALK-Abelló, Nieuwegein, the Netherlands) and a positive specific IgE test to peanut and/or soy (Phadia ImmunoCAP, Uppsala, Sweden) and/or a positive allergen provocation test were selected for inclusion ($n = 60$). After informed consent, 20 patients volunteered to participate in this study. IgE levels to peanut, soy and pea extract, rAra h 1, 2, 3 and 8 from peanut, rGly m 4, nGly m 5, nGly m 6 and 2S from soy (kind gifts of Sigrid Sjölander, Phadia, Uppsala, Sweden), rBet v 1 from birch and cross-reactive carbohydrate determinants (CCD) were determined by ImmunoCAP (Table 1).

Most (6/8) soy allergic patients had no specific IgE to soy extract or nGly m 5 and 6, but all patients showed IgE to rGly m 4 and four to soy 2S. Most (7/11) peanut, but clinically non-soy allergic patients, tested serologically positive for soy extract and for nGly m 5 and 6. IgE levels to peanut and soy extract were strongly correlated (Pearson $r = 0.924$, $P < 0.001$); and similar results were obtained for pea ($r = 0.821$, $P < 0.001$, data not shown). rGly m 4 was positive in four of the peanut allergic patients and its levels correlated in both soy and peanut allergic patients with IgE levels to rAra h 8 ($r = 0.917$, $P < 0.001$) and rBet v 1 ($r = 0.940$, $P < 0.001$). Cross-reactive carbohydrate determinants sensitization was negative for all patients except one (data not shown), which excludes the presence of cross-reactive IgE to legumes and grass pollen CCD.

Only rGly m 4 IgE levels were positive in all included soy allergic patients, which suggests rGly m 4 sensitization to be a valid indicator of severe soy allergy probably mediated by IgE cross-reactivity with Bet v 1 [5]. Thus Gly m 4 seems to be more relevant in detecting clinical soy allergy than nGly m 5, m 6, soy 2S or total soy extract. However, we cannot exclude a different soy sensitization profile in other countries. In our peanut allergic patients IgE sensitization to soy and pea extract was found to have low diagnostic specificity probably because of clinically non-relevant cross-reactivity between peanut specific IgE and homologous soy or pea components. Even a positive rGly m 4 test in peanut allergic patients could be clinically non-relevant as seen in 4 of 12 peanut allergic patients.

In conclusion, soy CRD seems to discriminate between solely soy allergic patients and soy and/or peanut allergic patients. However, especially in peanut allergic patients with IgE to Gly m 4, a soy challenge test is still indicated to confirm a clinically relevant soy allergy.

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Table 1 Clinical features and immunological findings of the peanut and/or soy allergic patients

Pt	Age/ sex	Symp. (grade); SPT (-/+/++)	Specific IgE to extracts (kU/l)		Specific IgE to recombinant peanut allergens (kU/l) (Percentage of total)								Specific IgE to soy allergens (kU/l)					Severity hay fever (-/+/++)	
			Peanut (f13)	Soy (f14)	rAra h1 (f422)*	rAra h2 (f423)†	rAra h3 (f424)‡	rAra h8 (f352)§	rGly m 4 (f353)§	nGly m 5 (f431)*	nGly m 6 (f432)‡	Soy 2S (f2s)†	rBet v 1 (f215)§	Tree pollen	Grass pollen				
peanut allergic patients																			
1	20/f	1; ++	0; nd	17.6	0.74	7.26 (43)	8.19 (48)	1.44 (9)	<0.35 (0)	<0.35	0.98	0.4	<0.35	<0.35	<0.35	-	-		
2	18/f	1/2; +	0; -	4.75	<0.35	2.32 (36)	4.21 (64)	<0.35 (0)	<0.35 (0)	<0.35	nd	nd	0.72	0.72	0.72	+	+		
3	25/m	2; ++	0; nd	33.2	0.61	6.34 (27)	11.5 (49)	5.65 (24)	<0.35 (0)	<0.35	0.47	0.85	<0.35	<0.35	<0.35	++	++		
4	22/f	2/3; ++	0; nd	33.6	1.13	9.42 (34)	16.3 (58)	2.17 (8)	<0.35 (0)	<0.35	0.73	1.63	0.58	0.59	0.59	++	-		
5	23/m	3; ++	0; -	8.59	<0.35	5.09 (76)	1.57 (24)	<0.35 (0)	<0.35 (0)	<0.35	<0.35	<0.35	<0.35	<0.35	2.77	+	-		
6	24/f	3; ++	0; nd	79.6	7.32	43.8 (60)	20.4 (28)	9.38 (13)	<0.35 (0)	<0.35	6.42	5.32	0.59	<0.35	<0.35	+	++		
7	27/m	3; ++	0; +	16	<0.35	5.45 (44)	5.13 (41)	1.92 (15)	<0.35 (0)	<0.35	<0.35	0.36	<0.35	<0.35	<0.35	-	#		
8	19/f	3/4; ++	0; nd	>100	7.29	98.5 (41)	>100 (42)	40.4 (17)	0.46 (0)	0.43	3.06	6.95	0.41	1.31	1.31	++	++		
9	18/m	4; ++	0; nd	>100	10.1	35.9 (27)	70.9 (53)	24.4 (18)	2.98 (2)	1.59	10.1	12.4	7.87	12.30	12.30	#	#		
10	46/f	3; nd	0; nd	54.4	0.76	6.94 (9)	50.7 (69)	<0.35 (0)	16.1 (22)	10.9	<0.35	0.46	0.43	25.70	25.70	-	+		
11	21/m	3/4; ++	0; nd	6.98	<0.35	5.04 (48)	2.57 (25)	<0.35 (0)	2.84 (27)	4.72	<0.35	<0.35	<0.35	13.00	13.00	+	+		
soy allergic patients																			
1	28/f	0; +	3; +	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	2.85	<0.35	<0.35	0.94	4.43	++	++		
2	51/f	0; +	3; -	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	1.17	<0.35	<0.35	<0.35	8.00	++	#		
3	58/f	0; +	3; ++	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	10.7	<0.35	<0.35	<0.35	41.20	+	+		
4	53/f	0; +	4; +	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	0.93	<0.35	<0.35	<0.35	<0.35	4.21	+	-		
5	54/f	0; +	4; +	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	7.05	<0.35	<0.35	<0.35	1.07	17.70	+	-		
6	60/f	0; +	4; ++	0.46	<0.35	<0.35	<0.35	<0.35	5.32 (100)	2.71	<0.35	<0.35	<0.35	0.39	15.70	++	++		
7	22/f	OAS; +	3; +	4.04	0.36	<0.35	<0.35	<0.35	6.15 (100)	6.45	<0.35	<0.35	<0.35	<0.35	25.70	++	++		
8	23/f	OAS; +	2/3; -	1.97	0.51	<0.35	<0.35	<0.35	19.1 (100)	14.4	<0.35	<0.35	<0.35	0.76	60.50	-	-		
peanut and soy allergic patient																			
1	21/f	3/4; ++	3; +	>100	6	35.8 (22)	>100 (63)	12.7 (8)	10.7 (7)	14	5.02	5.88	1.97	35.50	35.50	-	++		

Pt, patient; m, male; f, female; nd, not determined; Symp., symptoms; OAS, oral allergy syndrome; r, recombinant; n, native; Severity SPT: -, negative; +, mild positive; ++, highly positive; Severity grass and tree pollen allergy: -, negative; +, mild hay fever; ++, severe hay fever; #, positive SPT with unknown clinical relevance.

Symptoms grading is based on Brockow and Ring (6). Grade 1 = dermal symptoms; grade 2 = gastro-intestinal problems like nausea and or cramping; grade 3 = any of the former grades plus vomiting/diarrhoea and respiratory tract problems like throat pruritus or tightness; grade 4 = any of the former grades and respiratory arrest plus cardiovascular problems like hypotension.

*7S globulin; †2S albumin; ‡11S globulin; §PR-10 protein.

Chapter 7

Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells

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Abstract

Lactic acid bacterial strains have received interest for their immunomodulating activities and potential use in probiotic products. A wide variety of strain-dependent properties have been reported, but comparative studies on the species level are scarce. The objective of this study was to assess the immunomodulatory effect of *Lactobacillus* species on the cytokine profiles and proliferative response of human peripheral blood mononuclear cells (hPBMC), and in particular, on the comparison between the species *Lactobacillus acidophilus* and *Lactobacillus plantarum*. hPBMC from healthy donors were stimulated in the presence or absence of the lactic acid bacteria and cytokine production, surface marker staining, proliferation and cell death were determined after 1 and 4 days of culture. All *Lactobacillus* strains tested were capable of inducing the production of interleukin (IL)-1 β , IL-10, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α). The bacterial strains did not differentially influence the amount of proliferating, viable, apoptotic and necrotic cells. Generally, *L. plantarum* showed a significantly higher induction capacity of IFN- γ , IL-12 and TNF- α compared with *L. acidophilus*. We conclude that the variation in immunomodulatory effects between species is even larger than the variation between the strains of the same species. In addition we demonstrate that *L. plantarum* strains are most potent in skewing the T-cell differentiation toward a putative Th1 response.

Introduction

Strains of the genus *Lactobacillus* are increasingly being used as immunomodulating agents in probiotic products. Several clinical trials have demonstrated the health benefits of probiotic intake, and in many of these studies, immunomodulatory properties are key elements for the observed effects. These immunomodulatory studies using probiotics have mainly focused on inflammatory diseases of the gut and on allergic diseases, reviewed in Borchers *et al.* [1] and Ng *et al.* [2]. These studies generally confirm a large species and interstrain variation on the probiotic functionalities tested.

Frequently, the effects on the immune system are investigated *in vitro* by exposing human peripheral blood mononuclear cells (hPBMC) to bacteria or bacterial preparations. The capacity of lactobacilli to induce cell proliferation and stimulate proinflammatory or anti-inflammatory cytokines in coculture with hPBMC without affecting hPBMC cell viability, indicates that different strains of *Lactobacillus* may differentially stimulate immune cells. This signifies the ability of these bacteria to induce systemic effects that could modulate the immune response. Proposed immunomodulatory mechanisms include downregulation of local inflammatory responses through the modulation and subsequent expansion or induction of various subsets of T cells producing a collection of proinflammatory and anti-inflammatory cytokines. Alternatively, regulatory T cells could be induced by the activity of these bacteria and could be responsible for the systemically detectable immunomodulatory properties [3,4]. Understanding the bacteria-elicited cytokine patterns, and their systemic consequences, is of importance to develop better targeted applications for probiotic products.

It is recognized that the immunomodulating properties of lactic acid bacteria are strain

specific and that the effects of a specific strain must not be extrapolated to other strains [4,5]. Several studies have compared strains of different species within the genus *Lactobacillus* (e.g. [6-8]), but those studies have not compared species by including multiple representatives of the species. On the other hand, only a few studies have compared closely related strains and observed strain-dependent differences within a single species [5,9]. Studies on the comparative *in vitro* immunomodulatory capacities of different *Lactobacillus* species using multiple representatives within these species are, to the best of our knowledge, not available. *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus paracasei* are among the most predominant *Lactobacillus* species in the gut [10,11] and several strains within these species are marketed as probiotics.

Here, we have compared the *in vitro* immunomodulatory properties of *L. plantarum*, *L. acidophilus* and *L. paracasei*, both at the species level and at the strain level. Because *Lactobacillus casei* and *L. paracasei* are closely related taxa that are frequently confused and for which the type strains have been redesignated recently [12], we have included strains from either of these species and analyzed them as one group.

Materials and methods

Bacterial strains

A set of 51 *Lactobacillus* strains of the species *L. acidophilus*, *L. plantarum* or the *L. casei/paracasei* cluster were selected from our culture collections on the basis of high survival rates under conditions of low pH or the presence of bile, isolation from the gastrointestinal tract, or were strains from species that form the predominant *Lactobacillus* population in the gut. For subsequent experiments, five *L. acidophilus* and three *L. plantarum* strains were selected that were representative for the species with respect to cytokine induction (Table 1).

Table 1. Specification of the selected *Lactobacillus* strains

Species	Strain	Origin and/or source
<i>acidophilus</i>	CBI 66	Unknown
<i>acidophilus</i>	CBI 118	Unknown
<i>acidophilus</i>	CBI 237	Unknown
<i>acidophilus</i>	B1532	Rat feces
<i>acidophilus</i>	B228	Human feces
<i>plantarum</i>	B1836	Single colony isolate of NCIMB 8826 from human saliva
<i>plantarum</i>	B2261	Silage
<i>plantarum</i>	B2808	Human feces

Strains were cultured for 24 h at 37 °C in Man Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), after which fresh broth was inoculated with 1% (v/v) overnight culture. After an additional 24 h of incubation at 37 °C, bacterial cells were harvested by centrifugation at 1000 *g*, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial cell numbers were determined by plate counting on MRS agar, and OD_{600 nm} was meas-

ured. The suspension was sterilized for 15 min at 115 °C and stored at -40 °C.

Isolation and culture of hPBMC

hPBMC were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) from freshly collected buffy coats of healthy donors (Sanquin Blood bank of Nijmegen, the Netherlands). An informed consent was obtained before the sample collection, and the experiments performed were approved by the local ethical committee. The various experiments were performed with hPBMC from different blood donors ($n = 1-3$).

For the screening experiment of the 51 *Lactobacillus* strains one buffy coat was used and cells from the interphase were harvested, washed and cultured in 24-well plates at 1×10^6 cells per well in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 1% penicillin-streptomycin (all from Gibco-BRL, Paisley, Scotland). For all other experiments cells from the interphase were harvested, washed and cultured in 48-well plates at 1×10^6 cells per well in Yssel's medium that consisted of IMDM containing GlutaMAX (IMDM) supplemented with 1% penicillin-streptomycin and 1% human AB serum (all from Gibco-BRL) with additions according to the previously described procedures [13].

Stimulation experiments

On the day of the experiment, the heat-killed bacteria were thawed, suspended in the appropriate culture medium and added directly to the hPBMC culture. Except for a pilot experiment to identify the most suitable ratio of bacteria to the hPBMC cells, a 1 : 1 ratio was used throughout this study. Medium with hPBMC, but without bacteria, was used as a negative (medium) control. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cultured cells and culture supernatants were harvested after 1 and 4 days of culture and these supernatants were stored at -20 °C and overnight transferred to -80 °C before analysis.

Cell viability

Trypan blue exclusion test

hPBMC viability after isolation was determined using the standard trypan blue exclusion test (Sigma-Aldrich, St. Louis, MO). The total number of viable and nonviable cells was counted using a Bürker counting chamber, and the percentage of viable cells was calculated accordingly.

Annexin V/propidium iodide (PI) staining

Measurement of early apoptosis and late apoptosis/necrosis was performed by double staining with APC Annexin V and PI. Half a million hPBMC were washed and incubated with 2 µl Annexin V (BD Biosciences, San Diego, CA) in 200 µl binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂]. After an incubation period of 15 min, cells were centrifuged and the supernatant was disregarded. After addition of 200 µl binding buffer and 2 µl PI (1 mg mL⁻¹; Sigma-Aldrich), cells were analyzed on a flowcytometer (FACSCanto II, BD Biosciences). Cells that were negative for both Annexin V and PI were considered as viable cells. Annexin-positive but PI-negative cells were regarded as apoptotic cells and double-positive cells were regarded as necrotic.

Surface markers

Immunological phenotyping was performed on the cells at 1 and 4 days of bacterial stimulation or unstimulated medium control. Surface antigens were identified by the monoclonal antibodies: anti-human CD3, CD4, CD8, CD14, CD19, CD25, CD16, CD56, CD45RA, CD45RO and anti-human CD69 (BD Pharmingen, San Diego). Appropriate isotype-matched controls were included.

Three monoclonal antibody mixtures were used: (1) α -hCD3 (PE-Cy7), α -hCD4 (PE), α -hCD8 (APC), α -hCD25 (APC-Cy7); (2) α -hCD14 (APC), α -hCD16 (PE), α -hCD19 (APC-Cy7) and α -hCD56 (PE); and (3) α -hCD45RA (PE), α -hCD45RO (APC) and α -hCD69 (APC-Cy7). The procedure was performed according to the instructions of the manufacturer. Four-color flowcytometric acquisition was performed on a FACSCanto II (BD Biosciences), using the BD FACSDiva software. A gate was set to exclude debris, and the percentages of cells expressing CD3, CD4, CD8, CD25, CD16/CD56, CD14, CD19, CD45RA, CD45RO and CD69 were then calculated. The percentage positive cells was corrected for values of the isotype control.

Proliferation

Proliferation was studied by intracellular expression of the nuclear Ki-67 antigen (BD Pharmingen) by flowcytometric analysis. Cultured cells were collected 4 days after bacterial stimulation or unstimulated medium control. In each assay, 5×10^5 hPBMC were incubated with 100 μ l cytofix/cytoperm (BD Pharmingen) for 15–20 minutes on ice to fix and permeabilize the cells. Cells were washed twice with perm/wash buffer (BD Pharmingen) and incubated with an anti-Ki-67 PE antibody (or the matched isotype control) diluted in perm/wash buffer for 30 min on ice in the dark. Then, the cells were washed once again with the perm/wash buffer, resuspended in PBS and measured on the flowcytometer. Values are expressed as the percentage of stimulated cells positive for the Ki-67 mAb corrected for the percentage of stimulated cells that were positively stained by the isotype control.

Cytokine analysis

For the screening experiment of the 51 *Lactobacillus* strains in the supernatants collected after 1 day of culture the production of the innate cytokines tumour necrosis factor- α (TNF- α), interleukin (IL)-12p70, IL-6 and IL-10 was detected with enzyme-linked immunosorbent assay (ELISA), using commercially available ELISA kits from Bioscience (Cytosets, Bioscience Europe SA, Nivelles, Belgium). Values are expressed as pg mL^{-1} (average of strains within the same species \pm SEM) deduced from the OD of the standard curve. The sensitivity limits for all tested cytokines were below 3 pg mL^{-1} .

For the follow-up experiments, cytokine production by hPBMC was analyzed in the supernatants of cells cultured for 1 and 4 days. The production of the innate and adaptive cytokines IL-1 β , IL-4, IL-5, IL-10, IL-12p70, interferon- γ (IFN- γ) and TNF- α was detected using Cytometric Bead Array (CBA, BD Biosciences). All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen), and the procedure was performed according to the manufacturer's protocol. The detection limits according to the manufacturer were as follows: 1.1 pg mL^{-1} IL-1 β , 1.4 pg mL^{-1} IL-4, 1.1 pg mL^{-1} IL-5, 2.3 pg mL^{-1} IL-10, 2.2 pg mL^{-1} IL-12, 0.3 pg mL^{-1} IFN- γ and 0.7 pg mL^{-1} TNF- α . The samples were measured on the FACSCanto II, using FCAP software (BD Biosciences).

Statistics

For all tests, homogeneity of variance was assessed using Levene's test. Strains ($n = 3-19$) from the same species were treated as one group. A one-way ANOVA followed by a Bonferroni test was used in case of equal variances between groups and a Games-Howell test in case of unequal variances between groups to analyze data comparing more than two groups. To compare two groups, an independent-sample t -test was used. When $P < 0.05$, the difference was interpreted as significant. The statistical analysis was performed by using SPSS software (version 15.0, SPSS Inc., Chicago).

Results

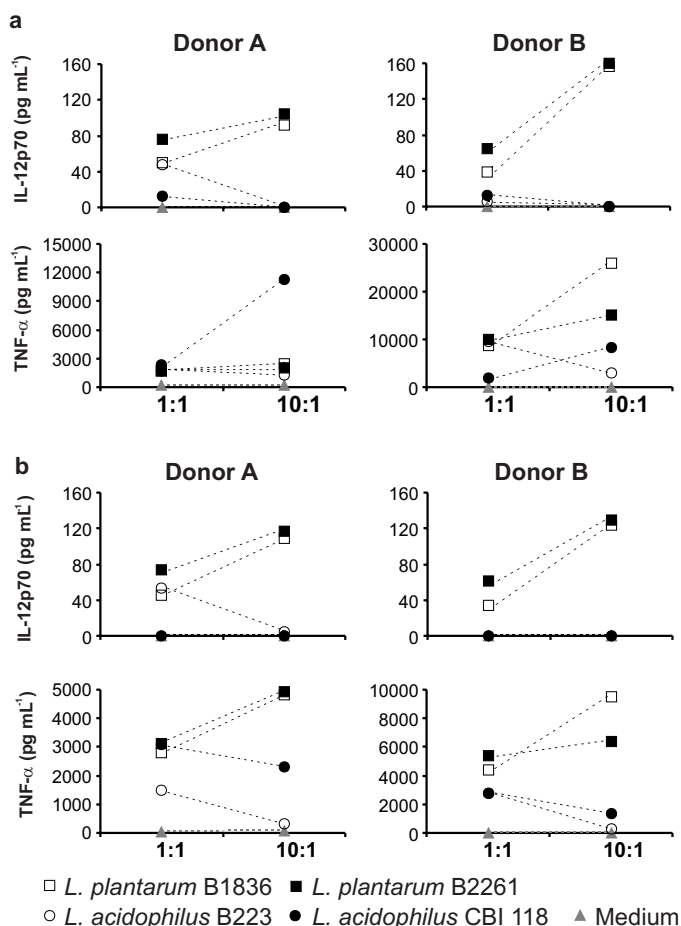
Comparison of *Lactobacillus* species with respect to cytokine induction in hPBMC

Using hPBMC of two donors, 10^6 and 10^7 bacteria, both in combination with 10^6 hPBMC mL^{-1} (bacteria : hPBMC ratio 1 : 1 or 10 : 1), were tested, and cytokine production was used as a read-out parameter. A medium control, two *L. acidophilus* (*L. acidophilus* B223 and *L. acidophilus* CBI 118) and two *L. plantarum* strains (*L. plantarum* B1836 and *L. plantarum* B2261) were included in this experiment.

In *L. plantarum*-stimulated hPBMC the IL-12 and TNF- α production in both donors and on both day 1 (Fig. 1a) and day 4 (Fig. 1b) were consistently up to fourfold higher using the higher dose of *L. plantarum* bacteria as compared with the lower dose. On the contrary, in *L. acidophilus*-stimulated hPBMC the IL-12 production was up to 10-fold lower or remained unchanged (with both bacterial concentrations tested below the detection limit) when stimulated for 1 and for 4 days with the higher concentration of *L. acidophilus* bacteria. Furthermore, the amounts of IL-12 in the supernatants detected on day 1 were strikingly similar to the amounts detected on day 4 and the variability between the two donors regarding IL-12 induction by the different strains is very low, underscoring the stability of this effect (Fig. 1). In both donors and with all tested *Lactobacillus* strains, IL-1 β and IL-10 production levels were consistently up to threefold higher when hPBMC were stimulated for 4 days with the lower dose of bacteria compared with the higher dose, while on day 1, this trend was not observed. Differences comparing the *L. acidophilus* strains with the *L. plantarum* strains were not observed regarding IL-1 β and IL-10 production. IL-4 and IL-5 levels were all below the detection limit (data not shown).

Based on the observed differences between the *L. acidophilus* and the *L. plantarum* strains particularly with respect to IL-12 induction capacity, a comparative study including 16 *L. acidophilus* strains, 19 *L. plantarum* strains and 16 *L. casei* and *L. paracasei* ssp. *paracasei* strains was performed. Selected strains were screened for their capacity to modulate the production of the cytokines IL-6, IL-10, IL-12 and TNF- α after an overnight stimulation of hPBMC. As shown in Fig. 2, IL-12 production was strongly increased in cultures stimulated with strains from the *L. plantarum* species ($127 \pm 12 \text{ pg mL}^{-1}$) compared with cultures stimulated with strains from the *L. acidophilus* species ($8 \pm 3 \text{ pg mL}^{-1}$; $P < 0.001$) or the *L. casei/paracasei* ($38 \pm 14 \text{ pg mL}^{-1}$; $P < 0.001$) species. IL-10 production in response to the species *L. casei/paracasei* ($372 \pm 90 \text{ pg mL}^{-1}$) was significantly lower compared with the *L. plantarum* species ($723 \pm 59 \text{ pg mL}^{-1}$; $P = 0.008$).

Fig. 1. Cytokine production of IL-12 and TNF- α after 1 day (a) and 4 days (b) of stimulation with two *Lactobacillus acidophilus* strains (circles), two *Lactobacillus plantarum* strains (squares) or the medium unstimulated control (triangles). Bacteria and hPBMC in concentrations of 10^6 bacteria and 10^6 hPBMC mL^{-1} (ratio 1 : 1) and 10^7 bacteria and 10^6 hPBMC mL^{-1} (ratio 10 : 1) were used. Results of two donors are shown: donor A (left panel) and donor B (right panel). The dashed lines connect the corresponding strains.



and also lower, although not significantly, compared with the *L. acidophilus* ($699 \pm 113 \text{ pg mL}^{-1}$; $P = 0.077$) species. The IL-10/IL-12 ratios were significantly enhanced for the *L. acidophilus* species (ratio 414 ± 224) compared with the *L. casei/paracasei* (ratio 22 ± 5) and the *L. plantarum* (7 ± 1) species. This observed difference in IL-10/IL-12 ratio is mainly linked to the enhanced IL-12 production of the *L. plantarum* strains. TNF- α production was significantly higher when hPBMC were stimulated with *L. plantarum* strains ($37252 \pm 545 \text{ pg mL}^{-1}$) ($27761 \pm 1864 \text{ pg mL}^{-1}$; $P < 0.001$ for *L. acidophilus* and $31152 \pm 1130 \text{ pg mL}^{-1}$; $P < 0.001$ for *L. casei/paracasei*). No significant differences were observed in IL-6 induction capacity between the three different species, with an average IL-6 production of the three species between 22000 and 25000 pg mL^{-1} (Fig. 2). The cytokine production values of the medium control were below the detection limit for IL-10, TNF- α and IL-6 and 3 pg mL^{-1} for IL-12.

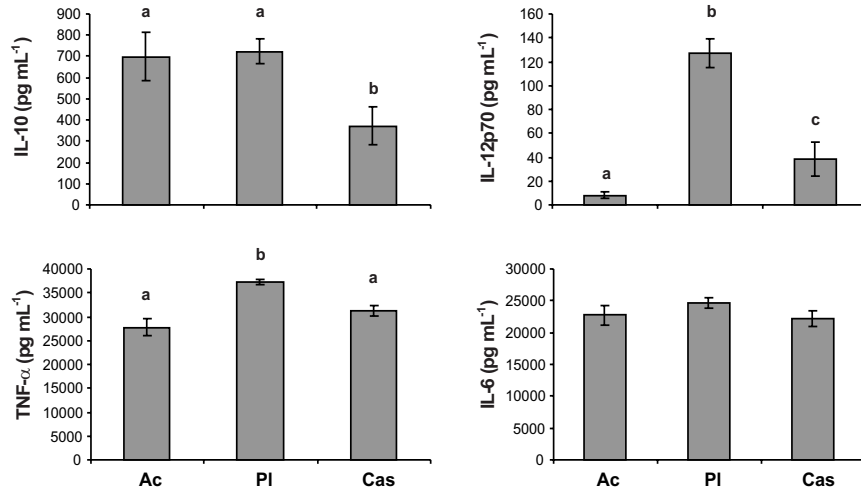


Fig. 2. IL-10, IL-12, TNF- α and IL-6 production by hPBMC isolated from one buffy coat and stimulated for 1 day with lactic acid bacteria from different *Lactobacillus* species. Concentrations of 10^6 bacteria mL⁻¹ and 10^6 hPBMC mL⁻¹ were used. The bars represent the mean cytokine production of all strains within one tested species. Ac, *Lactobacillus acidophilus* (tested 16 strains); PI, *Lactobacillus plantarum* (tested 19 strains); Cas, *Lactobacillus casei* and *Lactobacillus paracasei* ssp. *paracasei* (tested 16 strains). Error bars represent SEM. Means without a common letter differ ($P < 0.05$).

Effects of *L. acidophilus* and *L. plantarum* on hPBMC regarding viability and proliferation

Five *L. acidophilus* and three *L. plantarum* strains were selected for further studies, using three different blood donors, and the results obtained were consistent in all three donors tested. The selected strains represented the variation in IL-12 that was observed before in the larger screening experiment. The *in vitro* immunomodulatory effect of these eight selected bacterial strains on the response of hPBMC was investigated in more detail by assessing immune parameters other than cytokine induction. The viability of hPBMC directly after isolation was above 95%, and stimulation with *Lactobacillus* strains had no significant effect on the frequency of cells undergoing apoptosis or necrosis, either after 1 or 4 days of stimulation. After 1 day of culture, the average amounts of viable, apoptotic and necrotic cells were $90.0 \pm 1.9\%$, $7.0 \pm 1.4\%$ and $2.4 \pm 0.7\%$, respectively. At 4 days of stimulation, the average amounts of viable, apoptotic and necrotic cells were $73.3 \pm 1.3\%$, $16.1 \pm 1.3\%$ and $10.1 \pm 1.0\%$, respectively. The percentage Ki-67-positive cells at day 4 was below 5%, and independent of the presence of bacteria. In parallel experiments, cells were harvested after 8 days of culture and no difference was observed in the percentage of proliferation comparing unstimulated cells ($8.9 \pm 2.8\%$) with cells cultured in the presence of bacteria ($7.7 \pm 5.8\%$). Furthermore, no strain-related differences were observed (data not shown).

Effects of *L. acidophilus* compared with *L. plantarum* on hPBMC regarding the subset differentiation

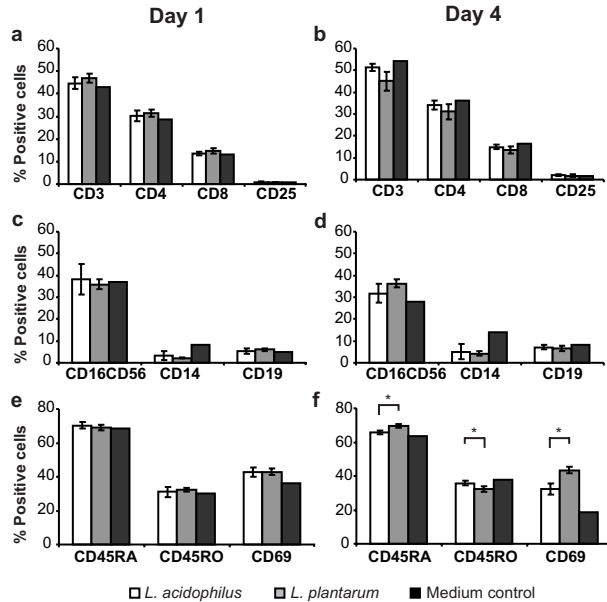
To assess the differentiation of the hPBMC into subsets and their activation status after stimulation with the different bacteria, a subset staining [CD3+ cells (T cells), CD4+ cells (T-helper cells), CD8+ cells (cytotoxic T cells) and CD25+ cells (activated cells)] was performed. In the medium control, only very low amounts of cells were activated (CD25+), and the addition of the different bacteria did not significantly change the T cell subset pattern nor the activation status of the T-cell subsets (Fig. 3a and b).

Figure 3c and d show the percentages of CD16+ CD56+ cells (NK cells), CD14+ cells (monocytes) and CD19+ cells (B cells) on day 1 and day 4, respectively. There were no significant differences in the percentages of NK, B or monocyte subsets between the *L. acidophilus* and the *L. plantarum* strains tested.

On day 4 of culture, the stimulation with *L. plantarum* strains induced significantly more CD45RA-positive cells (naïve cells) and fewer CD45RO-positive cells (antigen experienced or memory cells) compared with stimulation with *L. acidophilus* strains ($P = 0.009$ and 0.030 , respectively). On day 4 of culture, *L. plantarum* stimulation also showed more CD69-positive cells (early activated cells) compared with the *L. acidophilus* strains ($P = 0.001$). Furthermore, on day 4, the amount of CD69-positive cells was consistently higher in the cultures to which a lactic acid bacterial strain was added compared with the medium control (Fig. 3f).

Fig. 3. hPBMC were stimulated for 1 day (left panel) and for 4 days (right panel) with strains from the *Lactobacillus acidophilus* and *Lactobacillus plantarum* species. Culture medium was used as an unstimulated medium control. Concentrations of 10^6 bacteria mL^{-1} and 10^6 hPBMC mL^{-1} were used. hPBMC surface markers were stained with a mixture of α -hCD3, α -hCD4, α -hCD8 and α -hCD25 (a and b), α -hCD16/ α -hCD56, α -hCD14 and α -hCD19 (c and d) and α -hCD45RA, α -hCD45RO and α -hCD69 (e and f).

Statistical tests were performed comparing the *L. acidophilus*- and the *L. plantarum*-stimulated hPBMC cultures. Data are depicted as the average \pm SD. *Significant differences between the *L. acidophilus* and the *L. plantarum* strains ($P < 0.05$). Results are shown for one representative donor ($n = 3$).



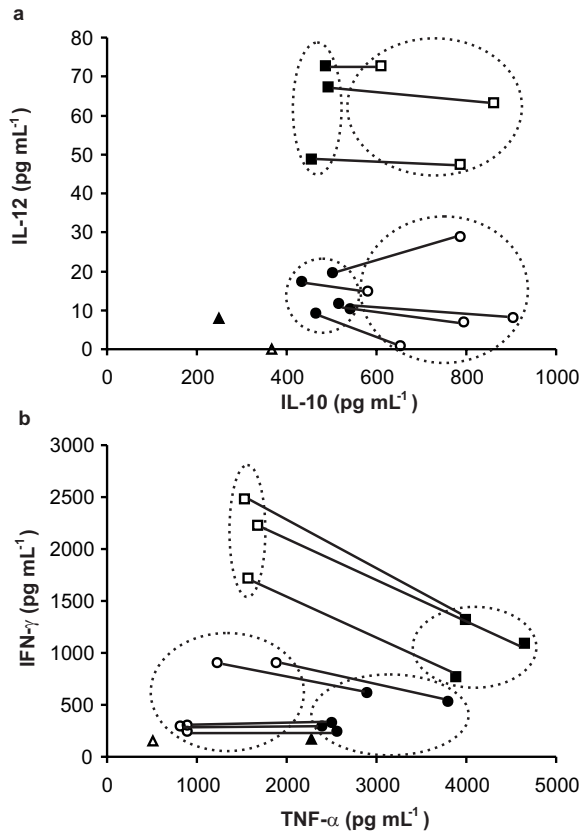


Fig. 4. Balance between IL-12 and IL-10 (a) and the balance between IFN- γ and TNF- α (b) production by hPBMC stimulated with bacteria of different *Lactobacillus* strains. Concentrations of 10^6 bacteria mL⁻¹ and 10^6 hPBMC mL⁻¹ were used. The circles represent the five *Lactobacillus acidophilus* strains, the squares show the three *Lactobacillus plantarum* strains and the triangles indicate the medium control. hPBMC were stimulated for 1 day (closed symbols) and for 4 days (open symbols). The lines connect the corresponding strains on the different days. The dashed circles indicate strains from the same species on a specific day and are placed to more clearly differentiate strains from different species and on different days of culture. Results are shown for one representative donor ($n = 3$).

Cytokine induction capacity profiles of the species *L. acidophilus* and *L. plantarum* after 1 and 4 days of stimulation

The production by hPBMC of IFN- γ , TNF- α (Fig. 4b) and IL-10 (Fig. 4a) after 1 (closed symbols) and 4 (open symbols) days of culture and of IL-1 β (Table 2) after 1 day of culture was induced upon interaction with all tested *Lactobacillus* strains (squares and circles) compared with the medium control (triangles). Comparing the IL-10 induction by the different species, no difference was observed in IL-10 induction capacity between the individual *L. acidophilus* and the *L. plantarum* strains. IL-10 production was significantly higher at 4 days compared with 1 day of stimulation ($P < 0.001$) (Fig. 4a).

A clear species-specific IL-12 induction was observed (Fig. 4a) both after 1 and 4 days of culture. *Lactobacillus plantarum* (squares) induced on average fivefold (2–11-fold) more IL-12 both after 1 and 4 days than *L. acidophilus* (circles) ($P = 0.015$ and 0.001 , respectively), and like in the previous experiment, whose results are shown in Fig. 1, the amounts of IL-12 detected after 1 day were similar to the amounts detected on day 4. The induction of the cytokine IFN- γ (Fig. 4b) by *L. plantarum* was on average fourfold (1.3–10-fold) enhanced compared with *L. acidophilus* after 1 day ($P = 0.005$) and after 4 days ($P = 0.001$) of stimulation. Furthermore, the production of the cytokine IFN- γ was on average two times higher at day 4 compared with day

1 ($P = 0.002$) in the cultures exposed to the *L. plantarum* strains. There was no significant increase in IFN- γ induction observed at day 4 compared with day 1 for the *L. acidophilus* strains.

As also observed in the initial screening experiment, the IL-10/IL-12 ratios were calculated for all strains and showed a significantly lower ratio for the *L. plantarum* strains (ratio 8 ± 1 on day 1 and 13 ± 4 on day 4) compared with the *L. acidophilus* strains (ratio 39 ± 13 on day 1 and 87 ± 52 on day 4) both after 1 ($P = 0.006$) and 4 ($P = 0.033$) days of stimulation (Fig. 4a). Again, this lower ratio is more linked to increased IL-12 production of the *L. plantarum* strains. Upon interaction with either *Lactobacillus* species, low or undetectable levels of the cytokines IL-4 and IL-5 (all below 5 pg mL^{-1}) were produced by the hPBMC (data not shown). Furthermore, no significant differences were observed in IL-1 β induction capacity after 1 or 4 days of culture between the two different species, although all strains increased the IL-1 β production by the hPBMC as compared with the unstimulated hPBMC after 1 day of culture, with at least 7400 pg mL^{-1} (Table 2). After 1 and 4 days of culture *L. plantarum* showed an increased induction capacity for TNF- α as compared with *L. acidophilus*; this finding was significant ($P = 0.012$) on day 1 but not significant ($P = 0.141$) on day 4 (Table 2). Furthermore, both IL-1 β and TNF- α production was significantly higher at 1 day compared with 4 days of stimulation (both $P < 0.001$).

Table 2. IL-1 β , IL-10, IL-12, IFN- γ and TNF- α production (pg mL^{-1}) by hPBMC stimulated with bacteria of five *Lactobacillus acidophilus* and three *Lactobacillus plantarum* strains.

	IL-1 β	IL-10	IL-12	IFN- γ	TNF- α		
Day 1							
<i>acidophilus</i>							
CBI 66	8763	267	4	79	287	hPBMC were stimulated for 1 day (upper table) or for 4 days (lower table). Concentrations of 10^6 bacteria mL^{-1} and 10^6 hPBMC mL^{-1} were used. Cytokine production values of the medium control are subtracted from each value. Medium values were as follows: IL-1 β , 7175 and 337 pg mL^{-1} ; IL-10, 249 and 368 pg mL^{-1} ; IL-12, 8 and ND pg mL^{-1} ; IFN- γ , 169 and 156 pg mL^{-1} ; TNF- α , 2272 and 508 pg mL^{-1} for day 1 and day 4, respectively. Results are shown for one representative donor ($n = 3$). *Significantly ($P < 0.05$) different compared with the average of the three <i>L. plantarum</i> strains on the same day. ND, nondetectable (below 2.2 pg mL^{-1}).	
CBI 118	14270	214	1	163	227		
CBI 237	7433	293	3	125	125		
B1532	8763	252	12	362	1518		
B228	11054	184	9	447	616		
Average	10057	242	6*	235*	554*		
SD	2691	43	5	160	569		
<i>plantarum</i>							
B1836	14270	242	60	922	2376		
B2261	11475	206	41	606	1615		
B2808	13769	238	65	1157	1715		
Average	13171	228	55	895	1902		
SD	1491	20	13	276	413		
Day 4							
<i>acidophilus</i>							
CBI 66	166	427	3	87	386		
CBI 118	74	285	ND	149	386		
CBI 237	101	536	4	137	304		
B1532	-177	419	25	747	1376		
B228	-54	213	11	747	718		
Average	22	376	8*	373*	634		
SD	137	127	10	342	444		
<i>plantarum</i>							
B1836	-69	492	59	2069	1171		
B2261	-48	419	43	1560	1061		
B2808	-123	242	68	2328	1026		
Average	-80	384	57	1986	1086		
SD	39	129	13	391	76		

Cytokine induction capacity profiles between strains

Next to differences at the species level, strains within a species differed in their potential to induce cytokine production as compared with each other. To illustrate this: after 1 and 4 days of culture, the strains *L. acidophilus* CBI 66, CBI 118 and CBI 237 showed the lowest IL-12, IFN- γ , and TNF- α induction of the five *L. acidophilus* strains tested. On the contrary, at day 4, these three strains showed the highest IL-1 β induction compared with the other two *L. acidophilus* strains, B1532 and B228. Addition of the latter two strains in the culture even caused a reduction in IL-1 β production compared with the medium control (Table 2). Furthermore, *L. acidophilus* B1532 is the most potent IL-12 and TNF- α inducer of the *L. acidophilus* strains. Regarding the *L. plantarum* strains, a similar production of IL-10 is seen for all *L. plantarum* strains on day 1, while on day 4 *L. plantarum* B1836 and *L. plantarum* B2261 induced a twofold higher IL-10 secretion compared with the *L. plantarum* B2808 strain (Table 2). Although strain-specific differences in immunomodulatory capacity clearly exist, differences between species are more profound. The cytokine induction capacity of the strains on day 1 differed within a species maximal 2.5-fold, while between the two species *L. acidophilus* and *L. plantarum*, the cytokine induction capacity differed up to eightfold.

Discussion

The study described here confirms and extends the strain-specific characteristics of different species of lactic acid bacteria in their immunomodulatory potential of hPBMC. This illustrates the strain specificity of candidate probiotic characteristics, and indicates that probiotic bacteria have to be selected with care, because immunomodulating capacity even differs between closely related strains. By comparing strains of lactic acid bacteria belonging to either *L. acidophilus*, *L. casei/paracasei* or *L. plantarum*, we demonstrate the species-dependent characteristics of *Lactobacillus* strains. Based on this improved insight into the characteristics of their immunomodulating activity, screening efforts to select new potential probiotic strains can benefit from a first focus on selected species rather than random screening of individual strains.

All *Lactobacillus* strains tested in our study stimulated IL-10, IFN- γ and TNF- α production both after 1 and 4 days of culture as well as IL-1 β after 1 day of culture. In support of our results, *Lactobacillus* strains are generally strong inducers of the proinflammatory cytokines TNF- α [14-16] and IL-12 [15,17-19], although several studies observed that specific lactic acid bacteria yield a low or nonsignificant increase in IL-12 production by hPBMC [7,20]. In our study, all *L. plantarum* strains tested stimulated IL-12 production, while most *L. acidophilus* strains failed to do so, which confirms that the difference in IL-12 induction observed in the reported studies is caused by the use of different species and strains. The data presented here indicate that strains belonging to *L. plantarum* have a higher capacity to induce innate cytokines, like IL-12 and TNF- α in cultured hPBMC than strains of *L. casei/paracasei* or *L. acidophilus*. Also, increasing the bacteria : hPBMC ratio increased the levels of IL-12 for *L. plantarum*, while *L. acidophilus* failed to do so. *Lactobacillus plantarum* induced on average more IFN- γ , IL-12 and TNF- α and therefore has more capacity to induce a putative Th1 subset response as compared with *L. acidophilus*.

Recent studies showed differences between *Lactobacillus* strains in their cytokine-inducing properties such as for IL-10 and IL-12, by comparing only a few *Lactobacillus* strains [6,7,16,17,21]. Furthermore, Miettinen *et al.* [15] observed a strain-specific effect on the Th1 cytokine induction capacity of three *Lactobacillus* strain on analyzing the cytokines IL-12 and IFN- γ . In the present study, initially, 51 strains were compared for their cytokine-inducing properties. The differential cytokine-inducing capacity within the *Lactobacillus* genus was used to select eight representative strains out of the *L. acidophilus* and *L. plantarum* species for further immunological characterization. To our knowledge, other studies have not determined interspecies differences in immune responsiveness assessing various immune parameters, despite the fact that several studies have reported strain-specific differences. In the present study, we also studied cell viability, proliferation, cell differentiation and activation of hPBMC by the various lactobacilli tested. No changes in cell viability or proliferation were observed after addition of the strains, which suggests that the stimulus provided is rather subtle, and that the bacteria by themselves do not promote lymphocyte survival nor induced immune proliferation *in vitro*. The observed increase in early activated cells (CD69) of the hPBMC stimulated with *L. plantarum* strains is significantly higher compared with the increase observed with the *L. acidophilus* strains. The performed double stainings showed that the CD69-positive cells are mostly naïve T cells (CD45RA), and together this finding is most consistent with an increased population of activated naïve T cells, despite the fact that B cells and NK cells also express this CD69 marker. This suggests that the *L. plantarum* strains have a more profound effect on both the monocyte and the T-cell fraction of the hPBMC, which could also explain the higher production of most cytokines analyzed here. IFN- γ production is also increased by addition of bacteria to the cultures [18,19], although such an induction has not always been observed [15,22]. IL-12 plays an important role in driving the immune response toward a Th1 direction, which, by the release of IFN- γ , counteracts Th2 activity *in vivo* [23]. The increase in IFN- γ could therefore be partly explained by the induction of IL-12 production by the lactic acid bacteria. Our results show a clear positive correlation between IL-12 and IFN- γ production.

The anti-inflammatory cytokine IL-10 is increasingly regarded as a regulatory cytokine; it is a potent inhibitor of IL-12 and may suppress the emergence of an unbalanced Th1 response. Although the IL-10 production in our study increased significantly over time, IL-12 and IFN- γ production remained the same or even also increased. Furthermore, a significant decrease in the IL-10/IL-12 ratio was observed in the *L. plantarum* strains compared with the *L. acidophilus* strains or the medium control after 1 day of stimulation. The decreased IL-10/IL-12 ratio in the presence of *L. plantarum* strains is mainly due to enhanced IL-12 production, which is consistent with the increased capacity to induce a T-helper 1 profile. The capacity of lactobacilli to stimulate proinflammatory or anti-inflammatory cytokines in coculture with hPBMC indicates that different *Lactobacillus* strains may differentially stimulate immune cells and could therefore even exert opposite immunomodulatory effects.

In addition to strain-specific analysis of the cytokine induction capacity, time-dependent and dose-dependent effects of cytokine release were observed in our hPBMC stimulation assay. Many studies conducted on the immunomodulatory capacity of lactic acid bacteria measured the cytokine production only after one particular time point of stimulation despite the notion that kinetics of production are cytokine-specific [22]. For example, Lammers *et al.* [24] found a time-dependent effect for *L. casei* and *Bifidobacterium breve* by exposing hPBMC for 24, 48

and 72 h to $20 \mu\text{g mL}^{-1}$ probiotic DNA. IL-1 β , IL-6 and IL-10 secretion reached the maximal concentration after 24 h and remained at this level. Chen *et al.* (1999) showed a time-dependent effect for IL-10 that reached the highest level in the supernatants at 24-h incubation, after which the production clearly decreased. Cytokine induction in hPBMC by viable probiotics also showed a concentration-dependent and time-dependent response. TNF- α production reached the highest induction after 24 h with 10^6 CFU mL^{-1} [25]. The kinetic analysis of the production level of multiple cytokines in our study provides a better overview of relevant innate and adaptive immune responses and will improve the understanding of the effects of lactic acid bacteria on cell differentiation, proliferation and cytokine production by the hPBMC.

The mechanism by which *Lactobacillus* species differentially stimulate hPBMC is largely unknown. Bacterial cell wall components are in direct contact with several immune cell types by binding various pattern recognition receptors, including the well-characterized family of Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain proteins. TLR usage by lactobacilli is species-specific, which is probably partly attributable to the considerable variation in the cell wall composition between the lactobacilli. For example, alterations of the cell wall polysaccharide-peptidoglycan complex composition of an *L. casei* strain have been reported to influence the cytokine responses of murine spleen cells and macrophages [26]. The TLRs identified as mediating certain activities of lactic acid bacterial strains include, among others, TLR2 and TLR9, reviewed in Borchers *et al.* [1]. Teichoic acids are considered to be one of the main immunostimulatory components of Gram-positive bacteria through their activation of TLR2 [27]. Teichoic acids are repeating units that are covalently anchored to either peptidoglycans (wall teichoic acids) or attached to the cytoplasmic membrane (lipoteichoic acids). There exist considerable differences between the wall teichoic acid and lipoteichoic acid molecules of different *Lactobacillus* strains, both in composition (e.g. the degree of substitution with D-Ala and/or glucose) and in quantity, which may influence the immune stimulating properties of the different strains [28]. Although all lactobacilli have teichoic acids in their cell walls, *L. casei* strains appear to contain only lipoteichoic acids whereas most *L. plantarum* strains contain the two types of teichoic acids, reviewed in Lebeer *et al.* [4]. Although the composition of cell wall structures forms a logical candidate for differential immunomodulating properties, the presence of other surface-associated proteins cannot be excluded. A recent study showed that the major S layer protein of the *L. acidophilus* NCFM strain is functionally involved in the modulation of dendritic cells and T-cells functions [29]. Furthermore, structural cell components such as pure genomic DNA and also to a minor extent secreted compounds of probiotics could also mediate part of the immune effects [1,5]. However, because the cells in the current study were washed and subsequently inactivated, it is not likely that secreted factors play a role, as demonstrated for other bacteria [30].

Investigations into the *in vitro* immunomodulatory capacity of probiotics usually precede the *in vivo* trials in animals or humans necessitating the existence of a good correlation between *in vitro* and *in vivo* findings. Nevertheless, systematic studies linking *in vitro* data to *in vivo* effects have rarely been performed [31]. This may be explained by the variety of parameters that interfere with such a comparison of strains including the bacterial preparations used (viability, growth phase, dose and timing of administration) [32], the method of hPBMC preparation, and the variable responsiveness of the donors. Nevertheless, the *in vitro* immune profiling in hPBMC with respect to the IL-10/IL-12 ratio induced by 13 bacterial strains is in-

deed predictive of their *in vivo* protective effect in a murine trinitrobenzene sulfonate model of acute colitis [31]. Whether this is true for inflammatory bowel disease in humans, or for other types of immune-related disorders, remains to be determined. Large numbers of candidate strains are often tested as probiotics for immunomodulating properties in a variety of *in vitro* models to select those strains with the best characteristics. A further selection takes place *in vivo* in animal models or human volunteers. Based on the study presented here, bacterial species can be classified for their probiotic characteristics on the basis of their immune profiles. Generally, *L. plantarum* strains were high IL-12 inducers, whereas strains from *L. acidophilus* were recognized as low IL-12 inducers. Depending on the aimed health benefit, this information would help to reduce the number of candidate strains to be tested.

In conclusion, in the present study, we have shown that several strains and species of lactic acid bacteria stimulate the T-cell response toward a putative Th1 response, which could be beneficial by downregulating a Th2-skewed disease such as IgE-mediated allergy. Especially, the *L. plantarum* strains, which, in our study, showed the highest IL-12 induction *in vitro* are promising candidates, as IL-12 plays a pivotal role in allergy by reducing the response of the Th2 pathway. To what extent probiotic *Lactobacillus* strains have differential *in vivo* immunomodulatory effects, especially in the field of allergy reduction that can be linked to the species level may be subject of further investigations. The immunomodulatory effects are shown to be species dependent, although the variation between strains is still of importance. This observed larger variation in the effects between species compared with within specific could be of practical value for the screening of new probiotic strains for their health-promoting properties.

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Chapter 8

***Lactobacillus* strains differentially modulate cytokine production by hPBMC from pollen-allergic patients**

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Abstract

The objective of this study was to assess the potential immunomodulatory effect of six *Lactobacillus* strains on human peripheral blood mononuclear cells (hPBMC) isolated from allergic patients. hPBMC from patients allergic to birch pollen or grass pollen were cultured *in vitro* in the presence or absence of selective bacterial strains. Cultures were left unstimulated or stimulated with α CD3/ α CD28 or Bet v 1. After 1, 4 and 8 days, cells and culture supernatants were harvested and the effect on cellular proliferation and the supernatant levels of several cytokines was assessed. All strains had the ability to repress IL-13 production but did show a differential effect on IFN- γ induction. Both strains B223 and B1697 showed a lower IFN- γ , IL-12 and TNF- α induction as compared with the other tested strains. Strain B633 showed the best proliferation-suppressive properties in α CD3/ α CD28-stimulated cells. Suppression of the T-helper type 2 (Th2) cytokine induction and induction of the Th1 cytokine production by specific strains might be beneficial for allergic patients having a disturbed Th1/Th2 immune balance. Furthermore, hPBMC of patients with seasonal allergy outside the pollen season can be used to determine immunomodulatory activities of probiotic bacteria.

Introduction

Atopic diseases such as allergic asthma, allergic rhinitis or allergic conjunctivitis, and atopic eczema have become an increasing health problem, and the use of probiotics appears to offer novel perspectives for treatment [1-5]. Lactic acid bacteria are well known for their practical application, while some lactic acid bacterial strains exert a beneficial effect on the host health and are therefore called probiotics. A variety of probiotic strains have been studied for their immunomodulating activities, including a selection of the 152 different species of the *Lactobacillus* genus that have been identified to date (NCBI taxonomy database), which encompass an unusually high phylogenetic and functional diversity [6]. It is recognized that each strain can have unique and markedly different immunomodulating properties. Consequently, the probiotic effects of a specific strain cannot be directly extrapolated to other strains of the same species, let alone across the species boundary [7-10].

An allergic reaction is characterized by a disrupted T-helper type 1 (Th1)/Th2 balance toward a preferential allergen specifically induced Th2 cytokine profile, causing allergic inflammation by releasing IL-4, IL-5, IL-9 and IL-13, inducing IgE antibody formation, promoting eosinophil development and recruitment, and increasing production of mucus in the gut and airways [11,12]. Suppression of Th2 and induction of Th1 cytokine production and induction of T-regulatory (Treg) cells could thus be beneficial in treating allergic diseases by antagonizing the Th2 cell development, resulting in suppressed IgE formation [13,14].

A proposed effect of probiotics is down-regulation of the Th2 cytokine production either by stimulation of Th1 cytokines or by stimulation of the regulatory cytokine IL-10, produced by antigen presenting cells such as monocytes [15-17]. Furthermore, the activities of Th1 and Th2 are suppressed via IL-10 and TGF- β production by Treg cells, to help in balancing the intestine [11,18-20]. Deficiency in functional Treg cells is currently widely accepted as a possible

mechanism underlying the Th2-skewed response in allergy [21,22]. Lactobacilli can upregulate the induction of Treg cells, triggering the release of regulatory cytokines and controlling the delicate balance between Th1 and Th2 immunity as well as tolerance [5,13,23,24]. The differential effects of probiotic strains are frequently investigated *in vitro* using human peripheral blood mononuclear cell (hPBMC) but generally derived from healthy donors [25-28], and only few studies have investigated the *in vitro* response of probiotics to hPBMC of allergic patients [15,17,29,30]. Healthy subjects, in contrast to allergic individuals, are assumed to regulate the Th1/Th2 balance by inducing sufficient Treg cell activity to maintain or restore immune tolerance to allergens [13,21].

The aim of the present study was to investigate the immunomodulatory capacity of six selected *Lactobacillus* strains and one mixture of two strains on hPBMC of pollen-allergic patients. Birch- and grass pollen-allergic patients were chosen as these are common seasonal allergies with a prevalence estimated up to 40% [31], and a possible benefit of probiotics could thus be of interest for a large part of the population. Human trials on probiotics have shown promising results for prevention of atopic eczema; however, the results on possible benefits for management of inhalant allergies, such as hay fever are not as conclusive [32,33]. Therefore, further research is needed to investigate whether probiotics can restore the disrupted immune balance in pollen-allergic patients, leading to a reduction of allergic symptoms.

The immunomodulatory properties of the selected *Lactobacillus* bacteria were assessed by measuring the induction of innate and adaptive cytokine production, proliferation and cell death of unstimulated, polyclonal stimulated and allergen-specific stimulated hPBMC. The *Lactobacillus* strains studied showed an overall stimulating effect on IL-10, decreased prototypical Th2 cytokines and differentially stimulated signature Th1 cytokine induction.

Materials and methods

Patients

Blood was collected from five birch pollen-allergic patients, two grass pollen-allergic patients and one adult healthy control. All birch- and grass-allergic patients reported having rhinoconjunctivitis during the birch or grass pollen season, respectively, and had serum-specific IgE to birch or grass pollen of at least class 4 (except for one person who had class 3), measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). The healthy donor displayed no birch or grass pollen-specific IgE in his sera ($< 0.35 \text{ kU L}^{-1}$ / class 0). Blood was obtained outside the pollen season in September, and none of the patients showed allergic symptoms at the time of investigation. Furthermore, none of the patients had received allergen-specific immunotherapy or used antihistamines or corticosteroids in the month before the blood drawing. All participants gave their informed consent and the performed experiments were approved by the local ethical committee (Commissie Mensgebonden Onderzoek, regio Wageningen).

Preparation of bacteria

Six *Lactobacillus* strains (Table 1) of the species *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus fermentum* were selected from our culture collection on the basis of high survival rates under conditions of low pH and/or the presence of bile, isolation from gastroin-

testinal tract, or were strains from species that are among the predominant *Lactobacillus* populations in the human gut. Further selection, including 70 strains, was based on IL-10-inducing capacities in 24-h hPBMC cultures of a healthy donor according to standardized procedures in our laboratories. Furthermore, a mixture of strains B2261 and B633 was included, further referred to as a mixture of B2261 and B633. The choice for this mixture was based on combining the highest IL-10-inducing strain (B633) and the highest IL-12-inducing strain (B2261), of the 70 strains included in this initial screening. Strains were cultured for 24 h at 37 °C in Man Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), after which fresh broth was inoculated with 1% (v/v) overnight culture. After an additional 24 h of incubation at 37 °C, bacterial cells were harvested by centrifugation at 1000 *g*, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial cell numbers were determined by plate counting on MRS agar, and OD was measured at a wavelength of 600 nm. The bacteria used in the immune-stimulation assays were heat-killed (15 min at 115 °C) and stored at – 40 °C until use.

Table 1. Specification of the selected *Lactobacillus* strains

Species	Strain	Origin and/or source
<i>L. plantarum</i>	B1836	Single colony isolate of NCIMB 8826 from human saliva
<i>L. plantarum</i>	B1697	Isolate from soak water soya beans
<i>L. plantarum</i>	B2261	Silage
<i>L. fermentum</i>	B633	Unknown
<i>L. acidophilus</i>	B223	Isolated from <i>Acidophilus</i> powder Laccillia
<i>L. acidophilus</i>	CBI 118	Unknown

Isolation, culture and stimulation of hPBMC

hPBMC were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) from freshly collected EDTA blood. Cells from the interphase were harvested, washed and cultured in 48-well plates at 1×10^6 cells per well in Yssel's medium, which consisted of IMDM-containing GlutaMAX (IMDM) (Gibco-BRL, Paisley, Scotland) supplemented with 1% penicillin-streptomycin and 1% human AB serum (all from Gibco-BRL) with additions according to previously described procedures [34].

On the day of the experiment, the heat-killed bacteria were thawed, suspended in the appropriate culture medium and added directly to the hPBMC culture in a 1:1 ratio with the cells. This ratio was identified as the most suitable ratio for this study as determined from a previous experiment [10]. To the culture exposed to the mixture of strains B2261 and B633, 0.5×10^6 bacteria of each strain were added to the 1×10^6 hPBMC per well. hPBMC were stimulated with α CD3/ α CD28 (150 ng mL^{-1} α CD3, 100 ng mL^{-1} α CD28), rBet v 1.0101 (Bet v 1; $10 \text{ } \mu\text{g mL}^{-1}$; Biomay) or left unstimulated. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cultured cells and culture supernatants were harvested after 1 day of culture without stimuli, after 4 days of culture without stimuli and with α CD3/ α CD28 stimulation. Cultured cells and culture supernatants were harvested after 8 days of culture of unstimulated and Bet v 1-stimulated cells, both with and without the addition of α CD3/ α CD28 as a restimulus on day 7. All supernatants were stored at – 20 °C and overnight transferred to – 80 °C before analysis. An overview of the *in vitro* experiments performed is presented in Fig. 1.

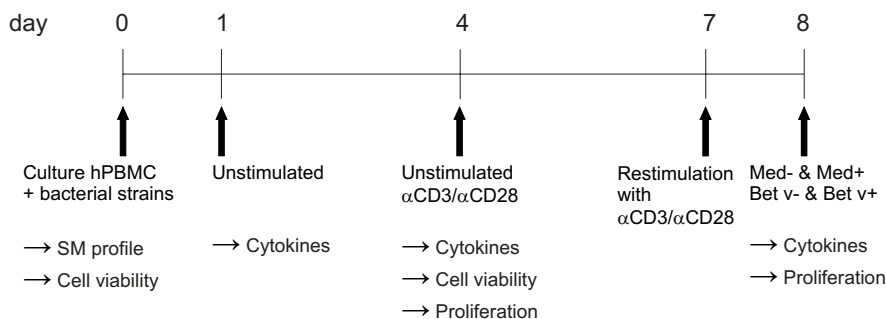


Fig. 1. Timeline of the *in vitro* study design. hPBMC were isolated from eight donors and cultured in 48-well plates in the presence or absence of the heat-killed bacteria and/or a stimulus. On the day of isolation, cell viability and the surface marker (SM) profile of the hPBMC of all eight donors tested were assessed. After 1 day of culture, supernatants were collected for cytokine analysis of unstimulated cells. On day 4, supernatants were collected for cytokine analysis of both unstimulated and α CD3/ α CD28-stimulated cells and cells were harvested to assess cell viability and proliferation. On day 7, α CD3/ α CD28 was added in the restimulated condition, and the next day supernatants were taken for cytokine analyses and cells were harvested to assess proliferation of unstimulated (Med-) or Bet v 1-stimulated cells, both without (-) and with (+) restimulation with α CD3/ α CD28 on day 7.

Annexin V/propidium iodide (PI) staining

Measurement of early apoptosis and late apoptosis/necrosis was performed by double staining with APC Annexin V and PI. Half a million hPBMC were washed and incubated with 2 μ l Annexin V (BD Biosciences, San Diego, CA) in a 200 μ l binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM CaCl_2]. After an incubation period of 15 min, cells were centrifuged and the supernatant disregarded. After the addition of 200 μ l binding buffer and 2 μ l PI (1 mg mL^{-1} ; Sigma-Aldrich) to the cell suspension, cells were analyzed on a flow cytometer (FACSCanto II; BD Biosciences). Cells that were negative for both Annexin V and PI were considered as viable cells. Annexin-positive but PI-negative cells were regarded as apoptotic cells and double-positive cells were regarded as necrotic. The Annexin V/PI staining was performed on cells immediately after isolation and on α CD3/ α CD28-stimulated cells after 4 days of culture in the presence or absence of the bacterial strains.

Surface markers

Immunological phenotyping was performed on cells immediately after isolation of the hPBMC. Two monoclonal antibody mixtures were used: (1) α -hCD3 (PE-Cy7), α -hCD4 (PE), α -hCD8 (APC) and α -hCD25 (APC-Cy7); (2) α -hCD14 (APC), α -hCD16 (PE), α -hCD19 (APC-Cy7) and α -hCD56 (PE). The procedure was performed according to the instructions of the manufacturer and the acquisition and analysis was performed as described previously [10].

Proliferation

Proliferation was studied by intracellular expression of the nuclear Ki-67 antigen (BD Pharmin-gen, San Diego, CA) by flow cytometric analysis. Cultured cells were collected on both 4 and 8 days of culture. In each assay, 5×10^5 hPBMC were incubated with 100 μ l cytofix/cytoperm (BD,

Pharmingen) for 15-20 min on ice to fix and permeabilize the cells. Cells were washed twice with perm/wash buffer (BD Pharmingen) and incubated with an anti-Ki-67 PE antibody (or the matched isotype control) diluted in perm/wash buffer for 30 min on ice in the dark. Hereafter, the cells were washed once again with the perm/wash buffer, resuspended in PBS and measured on the flow cytometer. Values are expressed as the percentage of stimulated cells positive for the Ki-67 mAb corrected for the percentage of stimulated cells that were positively stained by the isotype control.

Cytokine analysis

Cytokine production by hPBMC was analyzed in supernatants of cells cultured for 1, 4 and 8 days. The production of the innate and adaptive cytokines IL-1 β , IL-10, IL-12p70, IL-13, IFN- γ and TNF- α was detected using cytometric bead array (CBA; BD Biosciences). All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen) and the procedure was performed according to the manufacturer's protocol. The detection limits according to the manufacturer were as follows: 1.1 pg mL⁻¹ IL-1 β , 2.3 pg mL⁻¹ IL-10, 2.2 pg mL⁻¹ IL-12p70, 1.6 pg mL⁻¹ IL-13, 0.3 pg mL⁻¹ IFN- γ and 0.7 pg mL⁻¹ TNF- α . The samples were measured on the FACSCanto II, using FCAP software (BD Biosciences).

Statistics

Because of a nonnormal distribution of most of the data the nonparametric Wilcoxon signed-rank test was used. This test allowed to compare data from cultures in the absence of a bacterial strain with cultures in the presence of the different strains and to compare data from cultures of different strains. The Wilcoxon signed-rank test was also used to compare cytokine data on different days and to compare cytokine data on day 8 of not-restimulated and restimulated cells. When $P < 0.05$, the difference was considered to be statistically significant. The statistical analysis was performed using SPSS Software (version 15.0; SPSS Inc., Chicago). Experimental data are presented as mean \pm SEM.

Results

Composition of hPBMC subsets as measured directly after isolation

Although differences in hPBMC subset composition were observed between the different donors, all values were within the normal range of leukocytes present in the peripheral blood as assessed by Erkeller-Yuksel *et al.* [35] and Jentsch-Ullrich *et al.* [36] (data not shown).

Viability of hPBMC after isolation and after culture in the presence of *Lactobacillus* strains

Viability of hPBMC directly after isolation was above 80% for all donors and the percentage late apoptotic/necrotic cells was below 5% (data not shown). Furthermore, an AnnexinV/PI staining was performed after 4 days of α CD3/ α CD28-stimulated cultures. A slight but significant reduction of cell viability was observed in some but not all α CD3/ α CD28-stimulated cultures exposed to the bacterial strains compared with the control in which cells were stimulated with α CD3/ α CD28 in the absence of bacterial strains (Fig. 2).

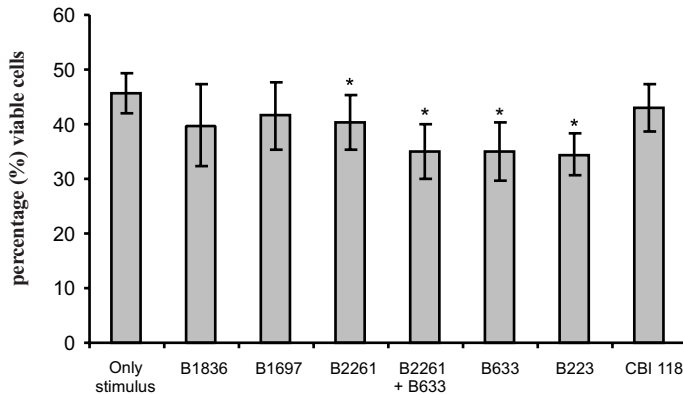


Fig. 2. Effect of lactobacilli on the percentage of viable cells in 4-day α CD3/ α CD28-stimulated cultures. Fresh hPBMC were stimulated for 4 days with α CD3/ α CD28 in the presence or absence (only stimulus) of a bacterial strain. hPBMC were stained with Annexin V and just before measurement, PI was added. All Annexin V-/PI-negative cells were regarded as viable cells. Data are mean \pm SEM values for seven allergic patients.

*Significant difference with the control group (only stimulus) in which no lactobacilli strain was added to the culture ($P < 0.05$).

Effects of lactobacilli strains on proliferation of hPBMC

To assess whether the different bacterial strains would have the ability to promote or repress the proliferation of hPBMC, the percentages of proliferating cells were measured for both α CD3/ α CD28-stimulated cultures and the long-term unstimulated or restimulated cultures exposed or not exposed to the different lactobacilli. The percentage Ki-67-positive cells after 4 days of culture that were not stimulated and without the addition of lactobacilli was below 5% (data not shown). As no effect was observed on the proliferation of hPBMC by the lactobacilli after 4 days of culture without an external stimulus [10], at day 4 in the current experiment, the Ki-67 staining was performed only for the α CD3/ α CD28-stimulated cultures. All lactobacilli significantly inhibited the proliferation induced by the polyclonal α CD3/ α CD28 stimulus (Table 2). Furthermore, strain B633 showed a significantly stronger inhibition of the proliferation compared with all other strains tested.

After 8 days of culture without an extra stimulus given on day 7, no difference was observed in percentage of proliferating cells on comparing hPBMC cultured in the absence of bacterial strains ($8.9 \pm 1.0\%$) with hPBMC cultured in the presence of the various bacteria (average of all bacterial strains $7.3 \pm 2.2\%$). Cells that were restimulated on day 7 with α CD3/ α CD28 showed a consistent inhibition of proliferation on day 8 in cultures to which lactobacilli were added compared with the control. An exception was strain B1697 which showed no or only a minor effect on the proliferation of hPBMC compared with control cultures, which were not exposed to a *Lactobacillus* strain. The observed inhibition of proliferation was significant for strains B2261, B633 and CBI 118 (Table 2).

Table 2. Proliferation of hPBMC in the presence or absence of lactic acid strains.

	Control	B1836	B1697	B2261	B2261 + B633	B633	B223	CBI 118
α CD3/ α CD28 day 4	40.2 \pm 3.5	32 \pm 3.4*	29.2 \pm 2.4*	30.7 \pm 2.5*	25.6 \pm 3.1*	9.4 \pm 1.8*	24.5 \pm 4.3*	26.9 \pm 4.0*
Medium -	8.9 \pm 1.0	6.9 \pm 2.1	7.2 \pm 2.5	7.4 \pm 2.0	6.4 \pm 1.9	6.9 \pm 2.1	7.9 \pm 2.0	8.4 \pm 2.9
Medium +	18.9 \pm 3.3	7.5 \pm 3.2	17.6 \pm 6.4	8.3 \pm 2.6*	8.8 \pm 3.5	6.5 \pm 3.4*	9.9 \pm 4.5	8.5 \pm 2.8*

Fresh hPBMC were stimulated with α CD3/ α CD28 for 4 days (α CD3/ α CD28 day 4), or cultured for 8 days and not restimulated (medium -) or restimulated (medium +) with α CD3/ α CD28 on day 7.

Data are mean percentage of Ki-67 positive cells \pm SEM values for seven allergic patients. Asterisks indicate statistically significant differences between control and bacterial species ($P < 0.05$).

Induction of cytokine production by the lactobacilli strains

The effect of the different lactobacilli strains on innate and adaptive cytokine induction of unstimulated hPBMC was investigated in cultures exposed to the lactobacilli but without addition of an external stimulus. IL-1 β production on day 1 (Fig. 3a) and TNF- α production on days 1 and day 4 (Fig. 3c) were induced upon interaction with all *Lactobacillus* strains tested. On day 4, both IL-1 β and TNF- α production were in all cultures significantly lower compared with that on day 1 (18- and 3-fold, respectively). Strains B1836, B1697 and B223 showed a higher IL-1 β induction compared with the control on day 4. IL-10 production was significantly induced for all strains and on both days compared with the control (Fig. 3b). Strains B1836, B2261, the mixture of B2261 and B633, and B633 alone induced a higher IL-10 production on day 4 compared with day 1.

Production of IFN- γ by hPBMC after 4 days of culture (Fig. 3d) was induced upon interaction with all *Lactobacillus* strains compared with the control. After 1 day of culture, IFN- γ production was consistently induced by all strains, except for strains B1697 and B223, and the IFN- γ induction was significantly higher on day 4 compared with that on day 1 (on average 16-fold). A clear difference in IFN- γ induction was observed for the different strains tested, with strains B1697 and B223 eliciting consistently low IFN- γ induction whereas the other strains were strong inducers. The strong IFN- γ -inducing strains also showed an increased IL-12 production, though IL-12 levels were, in all samples, below 25 pg mL⁻¹ (data not shown). IL-13 could not be detected on day 1 and was < 25 pg mL⁻¹ on day 4.

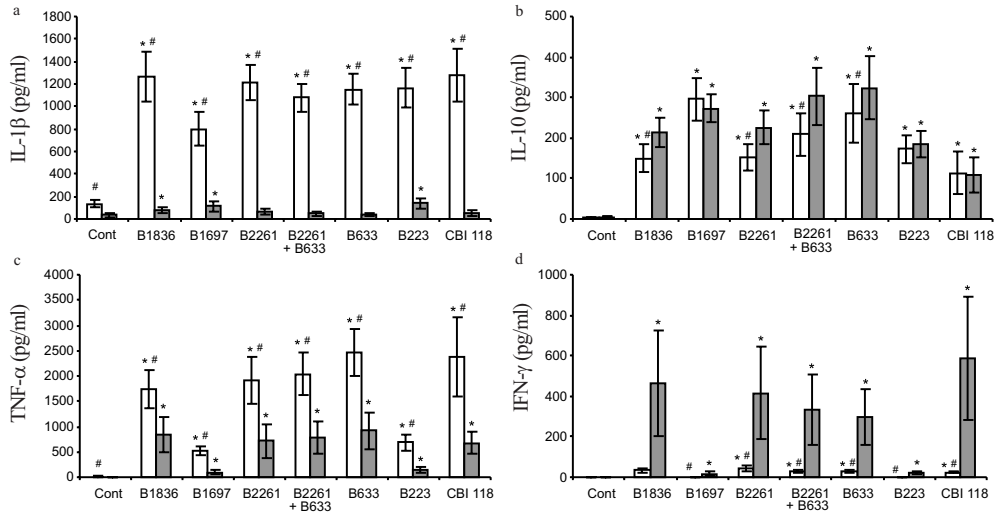


Fig. 3. IL-1 β , IL-10, TNF- α and IFN- γ production by hPBMC cultured for 1 day (white bars) and 4 days (gray bars) with lactic acid bacterial strains. The bars represent the mean cytokine production of all allergic donors tested ($n = 6$) and error bars represent SEM. *.#Significant difference with the control (Cont) group and day 4, respectively ($P < 0.05$).

To determine the effects of lactobacilli interacting with stimulated hPBMC, α CD3/ α CD28 was added to the culture and cells were cultured for 4 days. All strains inhibited IL-13 production by α CD3/ α CD28-stimulated hPBMC (Fig. 4f). Strain B2261, the mixture of strains B2261 and B633, and strain B633 alone were significantly stronger IL-13 inhibitors (on average a factor 7 inhibition) compared with the other strains tested (on average a factor 3 inhibition). There was a clear tendency of lactobacilli to inhibit IL-1 β production, except for strains B1697 and B223 (Fig. 4a), while TNF- α (Fig. 4c) and IL-10 (Fig. 4b) production was increased compared with the control for most strains, except for strains B223 and CBI 118. Addition of the various *Lactobacillus* strains to the hPBMC had no effect on IFN- γ production, which was high in all cultures after stimulation with α CD3/ α CD28 (Fig. 4d). IL-12 (Fig. 4e) was induced by strains B1836, B2261, the mixture of B2261 and B633, B633 alone and CBI 118, which was the same group of strains that also induced IL-12 and IFN- γ production in the unstimulated cultures.

The polyclonal stimulus α CD3/ α CD28 clearly induced IL-1 β , IL-10, TNF- α , IFN- γ and IL-13 production compared with the unstimulated cultures. The induction of IL-10 by the strains was significantly lower in the α CD3/ α CD28-stimulated cultures compared with the unstimulated cultures for the mixture of strains B2261 and B633, and strain B633.

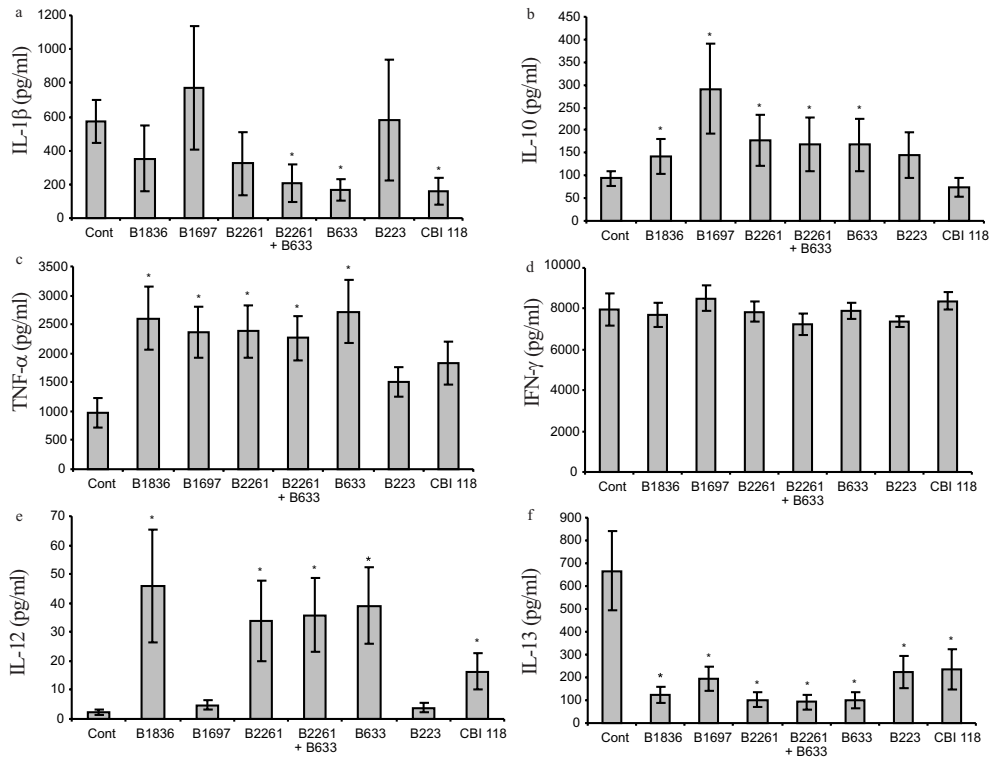


Fig. 4. IL-1 β , IL-10, IL-12, IL-13, TNF- α and IFN- γ production by hPBMC stimulated for 4 days with α CD3/ α CD28 in the presence or absence of lactic acid bacterial strains. Data are mean \pm SEM values for seven allergic patients. *Significant difference with the control (Cont) group to which no lactobacilli strain was added to the culture ($P < 0.05$).

Antigen-specific stimulation with the major birch pollen allergen Bet v 1

To determine the effect of the different lactobacilli on antigen-specific stimulated cultures, hPBMC of the five birch pollen-allergic patients were cultured in the presence of the major birch pollen allergen Bet v 1 and in the presence or absence of the different lactobacilli. After 8 days of culture, four stimulation conditions were compared. The restimulation condition with α CD3/ α CD28 on day 7 was used to increase the amount of antigen-specific T cells in the cultures which are still expected to be active in the culture and proliferate upon interaction with the specific antigen, Bet v 1. The addition of Bet v 1 did not result in significant differences in cytokine production profiles compared with the medium control. Only the IFN- γ production was higher in the Bet v 1-stimulated cultures compared with the respective medium control. The cytokine induction profile of medium compared with Bet v 1-stimulated cultures was similar and no Bet v 1-specific cytokine production could be detected (Table 3).

Table 3. Interleukin (IL) 1 β , IL-10, IL-12, IL-13, IFN- γ and TNF- α production by unstimulated or Bet v 1-stimulated hPBMC.

	IL-1 β	IL-10	IL-12	IL-13	IFN- γ	TNF- α
Med-	40.8 \pm 21.7	5.6 \pm 0.5*	0 \pm 0	131.4 \pm 42.8	32.7 \pm 20.2	5.2 \pm 2.1
Bet v 1-	52.3 \pm 18.1	10.3 \pm 3.5	0 \pm 0	94.8 \pm 26.8	13.4 \pm 1.6	2.9 \pm 0.5
Med+	109.4 \pm 38.6	389.0 \pm 47.6	29.1 \pm 16.4	2106.7 \pm 565.8	1498.4 \pm 352.4*	2258.1 \pm 640.9
Bet v 1+	133.7 \pm 26.3	372.7 \pm 48.0	15.5 \pm 10.0	1371.9 \pm 192.5	2797.0 \pm 623.8	3822.8 \pm 1504.7

hPBMC were stimulated for 8 days with medium (Med) or Bet v 1 (Bet v 1), both without (-) and with (+) restimulation with α CD3/ α CD28 on day 7. Concentrations of 10^6 bacteria mL $^{-1}$ and 10^6 hPBMC mL $^{-1}$ were used.

Data are mean \pm SEM values for five birch pollen-allergic patients. Asterisks indicate statistically significant differences between Med- and Bet v 1- or between Med+ and Bet v 1+ ($P < 0.05$).

Cytokine induction capacity profiles of the bacterial strains in long-term cultures

Cytokine production profiles were determined in the 8-day cultures without Bet v 1 both restimulated with or without α CD3/ α CD28 on day 7. This culture allows the detection of bacteria-induced modulation of accumulated cytokine levels in the supernatant. A significant inhibition of IL-1 β production was observed by strains B1836, the mixture of B2261 and B633, B633 and CBI 118 for both not-restimulated and restimulated cultures and also for strain B2261 in restimulated cultures compared with the respective controls (Fig. 5a and b). IL-12 production was low in both conditions, though similar effects of the various strains on IL-12 induction were observed as detected on day 4 with a low or even inhibited IL-12 production of strains B1697 and B223 (Fig. 5c and d). TNF- α induction capacity was increased in all not-restimulated cultures exposed to the various strains compared with the control, while in the restimulated cultures, most strains inhibited the TNF- α induction significantly (Fig. 5e and f). Furthermore, TNF- α was highly induced by the addition of α CD3/ α CD28 the day before harvesting the supernatants.

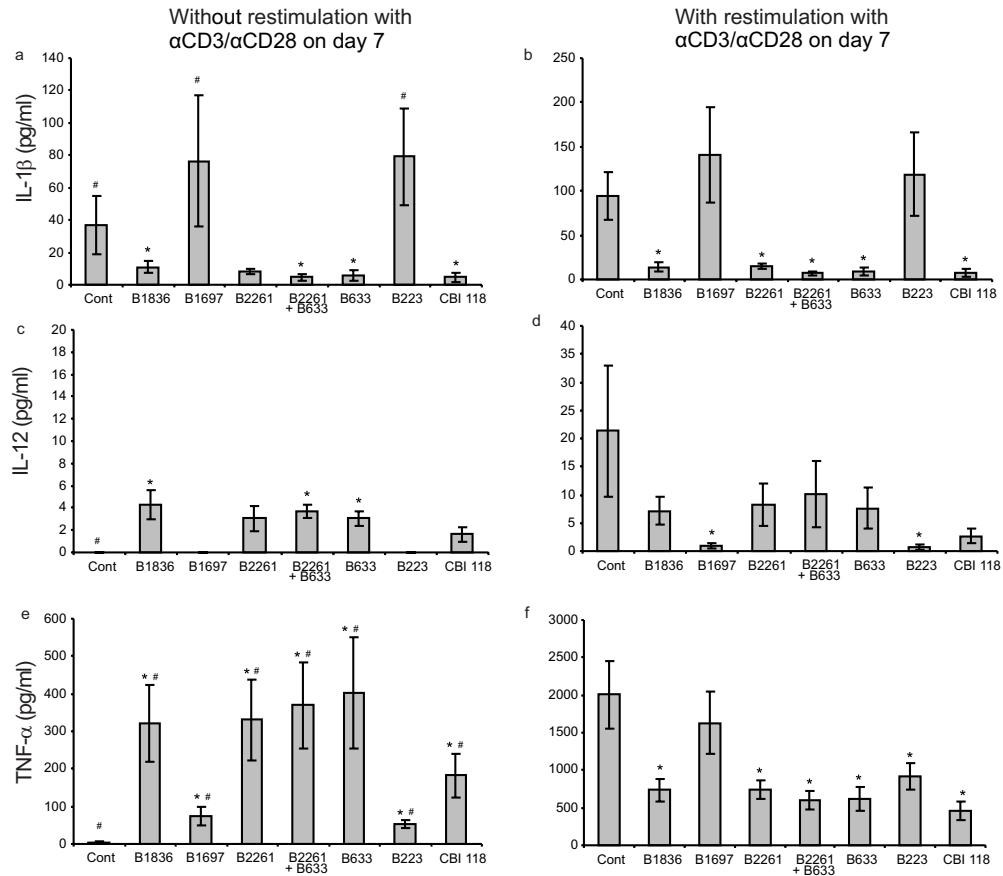


Fig. 5. Interleukin (IL) 1 β , IL-12 and TNF- α production by hPBMC cultured for 8 days in absence (Cont) or presence of lactic acid bacterial strains both without (left panel) or with (right panel) restimulation with α CD3/ α CD28 on day 7. Data are mean \pm SEM values for seven allergic patients.

*#Significant difference with control (Cont) group and day 8 with restimulation, respectively ($P < 0.05$).

In 8-day cultures of not-restimulated cells, IL-10 was significantly induced by all lactobacilli except for strain CBI 118 (Fig. 6a). In the restimulated condition, all strains significantly inhibited IL-10 induction capacity (Fig. 6b), and strains B1697 and B223 were significantly less strong IL-10 inhibitors compared with the other tested strains. Compared with IL-10 induction in 4-day α CD3/ α CD28-stimulated cells, the 1-day restimulation at day 7 induced a higher IL-10 induction. IFN- γ production was also induced by the restimulation on day 7 compared with not-restimulated cultures and effects of the strains were less prominent in the restimulated condition compared with the not-restimulated day 8 culture (Fig. 6c and d). IFN- γ production was induced by strains B1836, B2261, the mixture of B2261 and B633, B633 and CBI 118. Furthermore, IFN- γ production of unstimulated cultures was significantly higher on day 8 compared with day 4. After 8 days of culture of not-restimulated cells, IL-13 was consistently

decreased in the presence of the strains compared with the control, though this effect was not shown to be significant for strains B1697 and B223. This same inhibition was observed in the restimulated cells, and was significant for all tested strains. Strains B1697 and B223 were significantly less strong IL-13 inhibitors compared with the other tested strains. A clear induction of IL-13 production was detected by the restimulation with α CD3/ α CD28 on day 7 in the allergic patients (113 ± 40 pg mL⁻¹ for not-restimulated cultures vs. 1572 ± 488 pg mL⁻¹ for the restimulated cultures). This induction was much more clear in the allergic patients compared with the healthy control (56 for not-restimulated cultures vs. 204 pg mL⁻¹ for the restimulated cultures). However, the healthy control analyses also displayed a lower IL-13 induction in the cultures where a bacterial strain was present (on average 21 ± 2.8 pg mL⁻¹ in presence of a strain compared with 56 pg mL⁻¹ for the control).

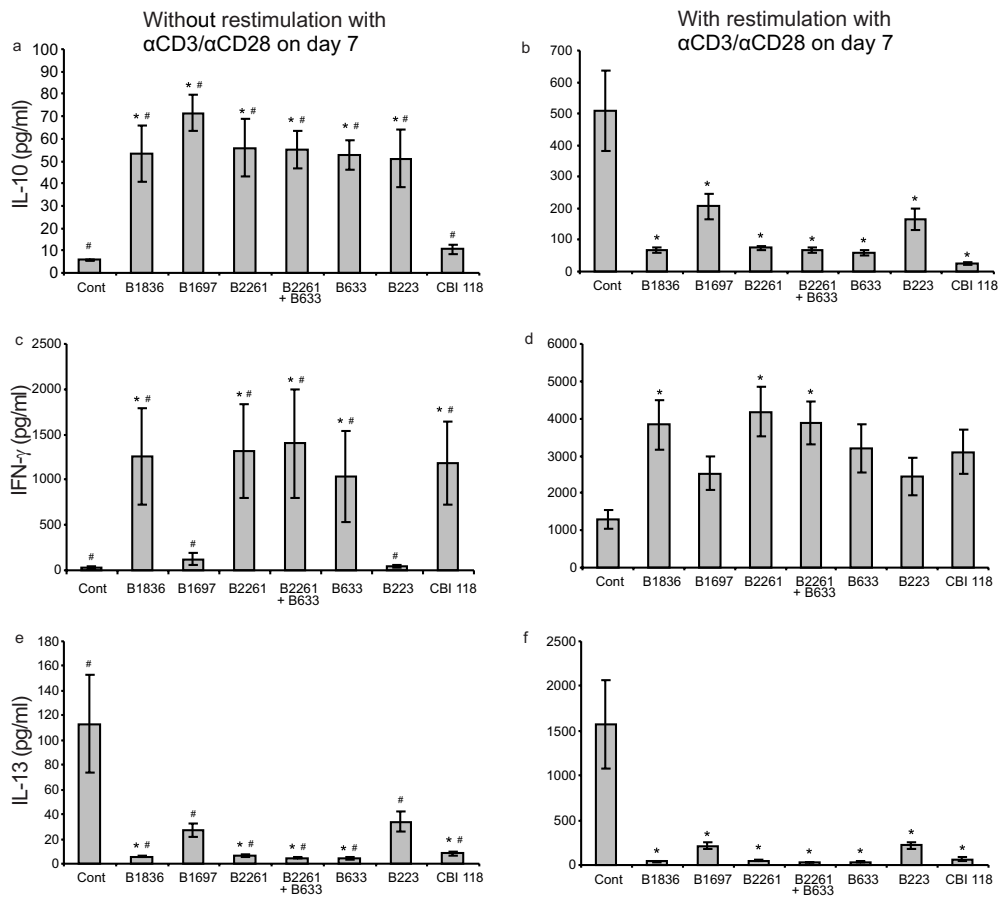


Fig. 6. Interleukin (IL) 10, IFN- γ and IL-13 production by human peripheral blood mononuclear cells (hPBMC) cultured for 8 days in absence (Cont) or presence of lactic acid bacterial strains both without (left panel) or with (right panel) restimulation with α CD3/ α CD28 on day 7. Data are mean \pm SEM values for 7 allergic patients.

*,#Significant difference with the control (Cont) group and day 8 with restimulation, respectively ($P < 0.05$).

The healthy control showed similar effects upon exposure of hPBMC to the different strains with respect to the cytokine induction profile. A difference compared with the allergic subjects was observed in the day 8 cultures that were not restimulated, as addition of the strains yielded higher IFN- γ values compared with the hPBMC cultures of the allergic patients. However, comparing the IFN- γ stimulation factor of the strains compared with the control, this factor was similar for the healthy control compared with the allergic patients (both around 35-fold). IL-1 β , TNF- α and IL-13 levels were lower in the healthy control compared with the allergic patients (results not shown).

Discussion

In this study, we aimed to determine whether different candidate probiotic strains of lactobacilli could *in vitro* modulate immune markers of patients with proven pollen allergy. Only few studies address the altered balance in the immune system of allergic individuals, and mostly include healthy subjects who are assumed to regulate their Th1/Th2 balance. We analyzed the capacity of lactobacilli to modulate this intrinsic capacity in allergic donors even out of the pollen season and to restore the T-cell balance in their immune system. The lactobacilli used here could be grouped into two categories based on their cytokine induction profile: a poor IFN- γ -inducing group, and a high IFN- γ -inducing group. This latter group, which also induced the regulatory cytokine IL-10, and strongly inhibited the release of the Th2 cytokine IL-13, might beneficially modulate the disturbed Th1/Th2 balance observed in allergic patients.

Culturing hPBMC for 1 day showed a clear induction of IL-1 β , TNF- α and IL-10 production by all strains tested, confirming the widely observed proinflammatory cytokine response induced by lactic acid bacteria. This response is presumably induced by monocytes as these respond rapidly after encountering bacteria or bacterial compounds by pattern recognition-mediated interaction [25,37,38]. While induction of IL-1 β and TNF- α are the highest on day 1, the induction of IL-10 is generally higher on day 4, which might indicate the contribution of T-cell subsets producing IL-10. IL-13 levels are low on day 1 and 4, but by day 8, all strains clearly inhibited the IL-13 induction compared with the control. The strong IL-13-inhibiting strains were found also to be strong TNF- α inducers. In a recent study, lactic acid bacteria were shown to have the ability to induce a proinflammatory cytokine response *in vitro* that was most likely insufficient to result in an inflammatory outcome upon ingestion of these bacteria by the allergic patients, but induced a higher state of the host innate defence system alertness [39].

With respect to the IFN- γ induction profile, a profound differentiation could be made between strong IFN- γ inducers (strains B1836, B2261, the mixture of B2261 and B633, B633 alone and CBI 118) and poor IFN- γ inducers (B1697 and B223). This differentiation has been observed for other strains [10,40,41], and was already detectable on day 1, being the most prominent on day 8. Activation of Th1 cells (rather than CD8+ T-cells and natural killer cells) is possibly responsible for this observation. Even though IL-12 production was low, IFN- γ induction and IL-12 production correlated on all tested days, as IL-12 and IFN- γ act synergistically. The differential cytokine activity profiles were also observed when comparing the IFN- γ /IL-13 ratio in the unstimulated day 8 cultures with strains B1697 and B223 having a 5 ± 3 and 30 ± 0 ratio, respectively, strain CBI 118 having a 188 ± 58 ratio and for all other strains, this ratio was be-

tween 250 and 355. This lower IFN- γ /IL-13 ratio for strains B1697 and B223 is mainly due to the lower IFN- γ induction and subsequent lower IL-13-inhibiting capacity.

The percentage nonviable cells of α CD3/ α CD28-stimulated cultures was in general higher than 50%, which is probably mainly caused by activation-induced cell death (AICD). AICD in T lymphocytes is the process by which cells undergo apoptosis in a controlled manner after activation through the T-cell receptor by, for example, CD3 monoclonal antibodies [42]. Furthermore, often the trypan blue exclusion technique is used to analyze cell death, in which early apoptotic cells will not be visualized and cell death numbers are, therefore, lower compared with the use of an AnnexinV/PI staining and flow cytometric analysis. The polyclonal α CD3/ α CD28 stimulus is widely used to provide all T cells with the required activation signals, with an optimum in proliferation and cytokine induction at days 3–5 [34]. This could explain why no difference was observed in IFN- γ induction between the control and the tested strains, as the effect of the strains is generally much weaker than the stimulus applied. However, the bacteria do have an effect on modulating this polyclonal stimulation with respect to some of the tested cytokines and proliferation, which strengthens the evidence that the bacteria can induce strong immunomodulating activities *in vitro*. The inhibition of IL-13 induction provoked by all tested strains was also observed for other strains in hPBMC cultures stimulated polyclonally using the lectin phytohemagglutinin [16] or the superantigen *Staphylococcus* enterotoxin A [15,17].

The lower IL-10- and IL-12-inducing capacity, together with the lower IL-13-inhibiting capacity of strain CBI 118 compared with the other high IFN- γ -inducing strains, could make this strain less favourable for use in allergic patients. A mixture of two strains from two different lactobacilli species (a combination of a high IL-10- and a high IL-12-inducing strain) was included in this study, but no clear synergistic effects were observed when compared with the individual strains. Although synergism is not always observed, some multispecies probiotic mixtures could expand the capacity for immunological modulation beyond that of the individual strains and might be effective in their immunomodulatory activity in selected patients [43–46].

Summarizing the differential cytokine production profiles of the tested strains, it was observed that specific strains selected on their IL-10-inducing properties, could be further grouped by their cytokine activity profile based on IFN- γ -inducing properties. The first group (represented by strains B1836, B2261, the mixture of B2261 and B633, B633 alone and CBI 118) induced a stronger proinflammatory TNF- α response, had a better inducing capacity of the Th1 compartment and showed a better inhibition of the Th2 cell compartment compared with the other group (represented by strains B1836 and B223), and might therefore be more appropriate candidate probiotics for allergic patients.

A remarkable finding was the consistent inhibition of proliferation of hPBMC stimulated for 4 days with α CD3/ α CD28 in the presence of all the applied bacterial strains with the most profound and significant inhibition by strain B633 in all seven allergic donors tested. However, addition of strain B633 did not impair cytokine induction, which strengthens the notion that various aspects of immunomodulation can be unique for a strain and that large species and strain differences exist in effects on inhibiting allergic inflammation, repression of hypersensitivity reactions and clinical symptoms of allergy [8,10,47]. These data support the notion that the probiotic potential of lactic acid bacteria as antiallergic compounds is strain-specific and largely variable already *in vitro* as is also reported upon *in vivo* use in randomized double-blind, placebo-controlled clinical studies [32,47].

Donors with a documented pollen allergy were recruited outside the pollen season, resulting in a low frequency of allergen-specific T cells that can be as low as 1 per 20 000 cells [48], consequently resulting in a low response to the Bet v 1 allergen. Enrichment of the allergen-specific T cells or the use of allergen-specific T-cell clones would be necessary to study potential modulatory effects of bacterial strains under allergen-specific culture conditions [49,50]. A significantly higher induction of IL-13 was detected in cultures restimulated on day 7 with α CD3/ α CD28 of the pollen-allergic subjects compared with the healthy control, which could indicate a higher number of Th2 cells in the culture in the pollen-allergic patients. Despite this, the lactobacilli inhibited IL-13 induction, regardless of donor, either allergic or not. In the long-term cultures and the α CD3/ α CD28-stimulated cultures, the increased IFN- γ and IL-12 secretion in hPBMC cultures exposed to the lactobacilli could mediate the Th2-suppressive effect, as observed previously [17,51]. However, the Th2 cytokine inhibition could be dependent on several parameters depending on the strains used [7,17,24].

The exact mechanism by which probiotic lactic acid bacteria modulate the host immune response is largely unknown. Bacterial cell surface macromolecules (such as long surface appendages, extracellular polysaccharides and teichoic acids) are in direct contact with several immune cell types by binding various pattern recognition receptors of the host. The structure of the main cell wall macromolecules is strongly conserved, but various modifications, such as glycosylation and also quantitative differences, can contribute to the strain-specific properties of probiotics. As little information is available regarding the specific bacterial components that for example induce the expression and production of cytokines, advances can be made in this area through the sequencing of genomes and transcriptomes that can be correlated to measured effects and enable testing which bacterial genes and derived components are essential to specific immunomodulatory properties [6,13,52-54].

Large numbers of candidate strains are often tested as probiotics for immunomodulating properties in a variety of *in vitro* models to select those strains with the best characteristics. In these *in vitro* studies, effects of heat-killed bacteria may not be directly extrapolated to effects of viable bacteria. Nevertheless, recent literature shows similar effects comparing live bacteria with heat-killed bacteria or even with components from the respective bacteria [15, 55, 56]. Very limited information is available with respect to the *in vivo* molecular responses to probiotic bacteria in human mucosal tissues; however, a recent study of van Baarlen *et al.* [57] showed a considerable overlap between *in vivo* human responses to live and heat-killed *L. plantarum*, provided that these bacteria were harvested from the same phase of growth. Systematic studies to link *in vitro* data to *in vivo* effects have rarely been performed so far and results are also not found to be consistent [58]. Based on the limitations of the *in vitro* model, extrapolations to *in vivo* effects must therefore be considered with caution.

Taken together, in this paper, the effect on probiotics on adaptive immune responses is included, by performing long-term cultures of hPBMC of allergic patients and analyzing typical T-cell-related cytokines and other read-out parameters. The present data clearly demonstrate that lactobacilli can modulate the cytokine induction profiles in hPBMC of allergic subjects *in vitro*. This modulation was most obvious in an increase in innate cytokine induction and a decreased synthesis of the Th2 cytokine IL-13 observed for all tested strains. Based on the present study, strains B1836, B2261, the mixture of B2261 and B633, and B633 alone could be chosen as most promising probiotic strains because of their stronger inhibition potential of IL-

13 induction and higher induction of IFN- γ and IL-12 compared with the other tested strains. Furthermore, the analysis presented here provides a suitable model to compare candidate probiotic strains for their immunomodulating properties *in vitro* in a Th2-skewed population and can even be used outside the pollen season, which makes this methodology a useful screening model.

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Chapter 9

Strain-specific immunomodulatory effects of *Lactobacillus plantarum* strains on birch-pollen-allergic subjects out of season

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Summary

Background: Allergic diseases are increasing world-wide, and according to the hygiene hypothesis may be related to a decreased exposure to environmental bacteria. Probiotic bacteria are recognized for their immunomodulating properties, and may benefit allergy patients. *In vitro* studies reveal immunomodulatory effects that are strain dependent. Differential immunomodulatory *in vitro* capacities cannot be extrapolated directly to *in vivo* efficacy. Thus, *in vitro* screening should preferably be followed by a comparative analysis of the selected immunomodulatory strains in an *in vivo* setting.

Objective: We selected five *Lactobacillus* strains on their IL-10-inducing capacity, and evaluated the immunomodulatory properties in birch-pollen-allergic subjects outside the hayfever season, with a reduction of IL-13 as the primary outcome.

Methods: A double-blind, placebo-controlled parallel study was performed in which 62 subjects with a proven birch-pollen allergy consumed one of five different probiotic yoghurts containing four *Lactobacillus plantarum* strains and one *Lactobacillus casei* strain or a placebo yoghurt. Blood samples were collected at the start and after 4 weeks. Several immune parameters were determined in serum and peripheral blood mononuclear cell cultures (PBMC) derived from these subjects.

Results: A decrease in birch-pollen-specific IgE was found for four probiotic strains. *L. casei* Shirota reduced the number of CD16⁺/CD56⁺ cells in peripheral blood mononuclear cells. For strain *L. plantarum* CBS125632, the decrease in IgE coincided with significant decreases in IL-5 and IL-13 production by α CD3/ α CD28-stimulated PBMC cultures.

Conclusion and Clinical Relevance: Subjects with seasonal allergy can be used to determine immunomodulatory responses outside the pollen season within a 4-week study period. *L. plantarum* CBS125632 decreased several immune markers related to allergy, and may have the potential to alleviate the severity of seasonal allergy symptoms.

Introduction

The prevalence of allergic diseases in the general population has increased alarmingly over the past 25 years, particularly in western industrialized countries [1]. According to the hygiene hypothesis, this increase in atopic diseases is due to a decreased exposure to microbial pathogens and changes in the intestinal microbiota [2-4]. Indeed, the microbiota of allergic children was found to harbour fewer lactobacilli as compared with non-allergic children [5]. However, the relation between microbiota and allergy development is likely more complex because a reduced diversity in the early fecal microbiota of infants with atopic eczema has also been reported [6]. This illustrates that the interplay between members of the microbiota and the host drives the development of a healthy immune system through mechanisms that are poorly understood.

Bacterial strains of the genus *Lactobacillus* are of interest as food-grade immunomodulating agents in so-called probiotic products. Probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host [7]. Several clini-

cal trials have demonstrated immunomodulatory properties of probiotics for the prevention or treatment of, e.g. inflammatory or allergic diseases [8]. Studies related to allergy have mainly focused on the use of *Lactobacillus* preparations for the prevention of early manifestations of allergic responses in newborns. Preparations given either to bottle-fed newborns or to breast-feeding mothers significantly reduced the risk of infants to develop atopic diseases [9,10], and in one study, this protective effect could still be observed in later life, even at the age of 7 years [11]. However, studies focused on allergy prevention in newborns do not necessarily relate to a reduction of symptoms in patients with an existing allergy. Indeed, in contrast to the promising effects on allergy prevention in newborns given *Lactobacillus rhamnosus* GG, no effect on birch-pollen allergy was observed when the same strain was given to adults [12].

Several, mainly, Japanese studies claimed that orally administered *Lactobacillus* strains had small beneficial effects on perennial allergic rhinitis [13] and on symptoms of Japanese cedar pollen allergy [14-17]. However, effects on immune markers like specific IgE levels or T-helper type 1/2 (Th1/Th2) ratio were not always reported, indicating that underlying mechanisms remain unclear and may include other than immunomodulatory explanations. In a recent pilot study, *Lactobacillus casei* Shirota showed a significant reduction in levels of relevant cytokines in grass-pollen-allergic subjects that coincided with an increase in specific IgG and a decrease in specific IgE [18]. Although the study period included the grass pollen season, no effects on the severity of symptoms were observed.

It is recognized that the immunomodulating properties of lactic acid bacteria are largely strain-specific. IgE-mediated allergy is the result of a Th2-mediated immune response and is characterized by the production of high levels of interleukins IL-4, IL-5 and IL-13 that promote the formation of IgE-producing cells [19]. A frequently proposed strategy to counterbalance a high Th2 response is the promotion of cells that secrete IL-10 [20-22]. **A widely accepted approach** to investigate effects of bacterial strains on the immune system is the *in vitro* exposure of human cells to bacteria or sub-cellular bacterial preparations, followed by cytokine measurements in the culture supernatant. This has led to the recognition that candidate probiotics have *in vitro* effects that are highly strain specific, although differential effects also occur at the bacterial species level [23]. Such studies demonstrate the potential of the bacteria used to modulate the immune response, but a straightforward extrapolation to the *in vivo* effects after oral administration of these bacteria is impossible [24]. Therefore, elucidation of probiotic functionality should preferably be based on *in vivo* validation of multiple strains, to validate the proposed immunomodulatory capacities observed *in vitro*.

Function screening of candidate probiotic strains is an important but time consuming and costly aspect of the selection process. Selection of probiotic strains is usually based on *in vitro* screening only, in several cases supported by validation in animal models. There are numerous reasons why the outcome of these studies may not be similar to human trials, e.g. limitations due to the simplicity of an *in vitro* model, poor survival of the probiotic strain in the human gut or heterogeneity in the human population [25]. Because of high costs, clinical trials are mostly performed with just one probiotic product, which has the risk that not the most appropriate strain is studied. Currently, there is no good *in vivo* selection model that evaluates the best candidates from *in vitro* screening before clinical testing occurs. In such an approach, it is of importance to use relatively small groups of subjects in order to screen a maximum number of strains, although conclusions should still be based on statistically sound evidence. Here,

we present a human screening system in which a limited number of pre-selected candidate probiotics can be evaluated. The effect of probiotics on birch-pollen-allergic subjects with a Th2-skewed immune system was followed outside the hayfever season during which exposure to birch pollen is highly unlikely. Especially IgE is sensitive to changes due to the relatively short half life of 2-3 days for this immunoglobulin class [26]. Without exposure to birch pollen, one would expect that antigen-specific immunity is relatively stable. In addition, pollen-specific memory Th cells decrease after the pollen season, but 60% survive up to 8 months [27]. Therefore, we hypothesized that birch-pollen-allergic subjects outside the hay fever season form a good read-out system for short-term immunomodulating interventions. Here we present a parallel study in which five probiotics selected for their high IL-10-inducing capacity and a placebo group were compared for their immunomodulating properties in birch-pollen-allergic subjects. Parameters of the study included changes in immunoglobulins, and cytokine secretion by peripheral blood mononuclear cells (PBMC).

Materials and Methods

In vitro selection of strains

Candidate probiotic strains were selected from our culture collections, and used to determine the *in vitro* IL-10-inducing properties as described previously [23]. Human PBMCs were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) from freshly collected buffy coats of healthy donors (Sanquin Blood Bank of Nijmegen, Nijmegen, the Netherlands). Cells were washed and cultured in 24-well plates at 1×10^6 cells per well in RPMI 1640 medium (Gibco-BRL, Paisley, Scotland) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine and 1% penicillin-streptomycin (all from Gibco-BRL). Heat-killed bacteria were dissolved in the appropriate culture medium, and added directly to the PBMC culture in a 1:1 ratio. The production of IL-10 was detected in supernatants collected after 1 day of culture using a commercially available ELISA kit (Cytosets, Biosource Europe SA, Nivelles, Belgium). A total of seven assays were performed in which a variety of strains were included with PBMC from different donors. Some strains were repeatedly tested in all assays, whereas others were included only in one or a few assays.

Patients and study design

Ethical approval for this double-blind, placebo-controlled parallel study was given by the local ethics committee (Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen, Nijmegen, the Netherlands). The trial was registered at ClinicalTrials.gov (registration identifier NCT01137357). The study occurred during the period November-December 2008 at the Rijnstate Hospital in Arnhem, the Netherlands. Candidate subjects with birch-pollen allergy were selected from the outpatient allergy clinic. The main inclusion criteria were age 18-50 years; a clinical history of significant, troublesome, symptoms of seasonal allergic rhinoconjunctivitis with/without asthma of a duration of at least 2 years during the birch-pollen season; positive (wheal diameter ≥ 3 mm) skin prick test to birch pollen (Soluprick[®] SQ; ALK-Abello A/S, Hørsholm, Denmark) and with specific IgE (\geq CAP allergy, class 2) to birch pollen. A total of 658 candidate subjects were sent a written invitation letter to participate in the study. Exclusion

criteria were use of probiotics during the last month before the trial, use of medication to influence the immune system, use of antibiotics in the last 2 months before the start of the trial, pregnancy, lactose intolerance, and blood values suggesting an ongoing infection at the start of the study. The first 62 positively responding subjects who met the selection criteria were included in the study, and stratified over 6 groups of 10 or 11 subjects (five experimental groups and one placebo). In order to balance groups as much as possible, the allocation was based on gender as the primary criterion, and the most recent data on severity of birch-pollen sensitivity in their medical record as the second criterion. The number of patients per group was based on an average of 30% reduction of IL-13 as the primary outcome. The person that formed the groups was not involved in the further study. The subjects were kept blind to probiotic assignment. All subjects were given a subject number for the study and received separately packaged fresh yoghurt drinks containing the same number. They were instructed to take one portion of the product each day for a period of 4 weeks. Consumption of any other yoghurt products during this period was not allowed. After the intervention period, the subjects' remarks on the consumed product, medicine usage and other allergy-related information (if any) were collected in a short questionnaire. At both the start and end of the intervention period, blood samples were collected and used to determine relevant markers (Fig. 1). The investigators were kept blind to probiotic assignment of the participants until all measurements were completed.

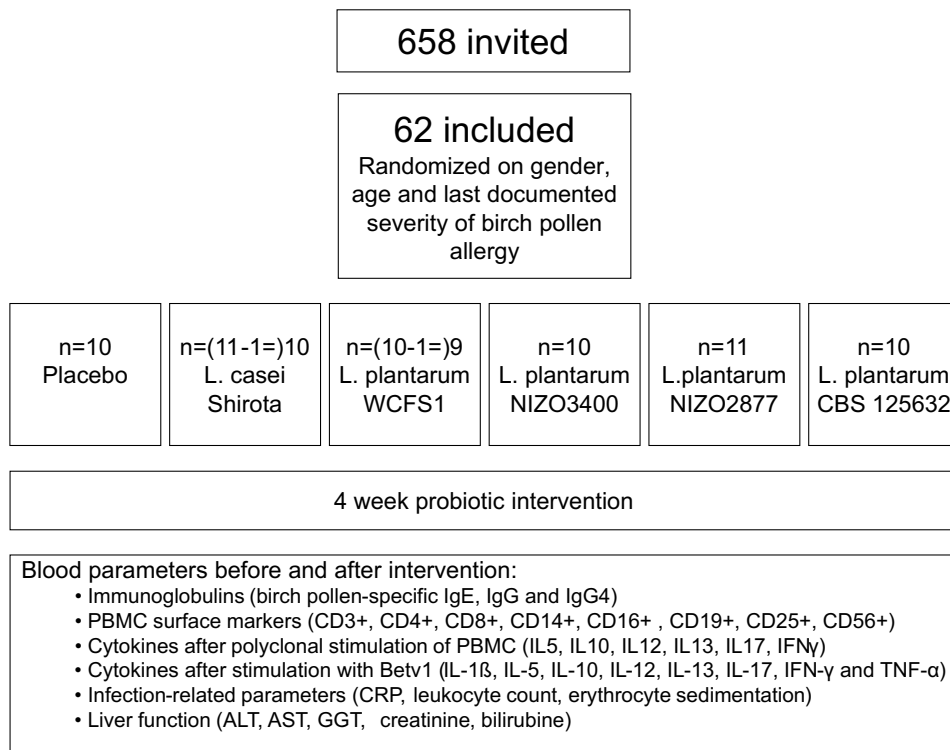


Fig. 1. Overview of the experimental design and tested parameters.

Probiotic or placebo drinks

Yoghurt drinks were prepared by inoculating pasteurized skimmed milk with a commercial yogurt starter (placebo) and in addition one of the following strains: *L. casei* Shirota, *Lactobacillus plantarum* WCFS1, *L. plantarum* NIZO3400, *L. plantarum* NIZO2877 or *L. plantarum* CBS125632. Milk fermentation by the product-specific lactic acid bacterium consortium was carried out for 10-14 h to reach a final pH of 4.2-4.3, followed by the addition and mixing of 5-15% lemon-lime flavoured syrup. The mixture was subsequently pumped, sheared, cooled and packaged in portions of 100 mL, each containing ca. 10^{10} CFU of the probiotic strain per portion. The probiotic and placebo drinks were identical in packaging, appearance and composition, except for the presence or absence of a probiotic strain. Subjects were instructed to store the product in their refrigerator and consume one bottle each day. To further assure the viability of the probiotic strains, the products were distributed on two occasions and consumed within 3 weeks after production. Stability of non-distributed test products was followed, and \log_{10} reduction in time was < 0.30 for all yoghurts.

Isolation and culture of human peripheral blood mononuclear cells

A total of 50 mL blood per time-point was collected in K-EDTA-coated tubes (for PBMC), lithium-heparin tubes (for automated blood cell differentiation) and uncoated tubes (for serum collection). PBMCs were isolated by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences) from freshly collected EDTA blood of the volunteers. We used a consistent way of isolation and cryopreservation of PBMCs in our experiments according to a protocol developed in our own laboratory as described previously [28]. Cells from the interphase were harvested, washed and frozen, and thereafter stored in liquid nitrogen until analysis. For cell-stimulation experiments, the cryopreserved samples were thawed by rapidly immersing them in a 37 °C water bath until they could be decanted. The cell suspension was transferred to a 50 mL polypropylene tube containing a 10 times larger volume of IMDM (Gibco-BRL) + 20% FCS. The cells were washed twice with IMDM + 20% FCS and then resuspended in Yssel's medium that consisted of IMDM-containing GlutaMAX (IMDM) supplemented with 1% penicillin-streptomycin and 2% human AB serum (all from Gibco-BRL) with additions according to previously described procedures [28]. Cells were cultured in 48-well plates at 1×10^6 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO₂. The supernatants were stored at -20 °C and overnight transferred to -80 °C before analysis.

Direct blood and serum analysis

The erythrocyte sedimentation analysis was carried out using the StaRRsed Compact Blood Sedimentation Instrument (Sysmex, Kobe, Japan) according to routine procedures. C-reactive protein, bilirubin, creatinine, ALT, AST and GGT were analysed using automated clinical chemistry analyzers (Roche, Basel, Switzerland).

Peripheral blood was centrifuged at 800 g for 10 min at room temperature, and the serum was removed for storage at -80 °C until analysis. Birch-pollen-specific IgE, IgG and IgG4 antibodies were determined using ImmunoCAP (Phadia AB, Nieuwegein, the Netherlands) according to the instructions of the manufacturer.

Surface markers

Immunological phenotyping was performed on the cells after thawing. Surface antigens were identified by the following monoclonal antibodies: anti-human CD3, CD4, CD8, CD14, CD19, CD25, CD16, and anti-human CD56 (BD Pharmingen, San Diego, CA, USA). Appropriate isotype-matched controls were included.

Two monoclonal antibody mixtures were used: (1) α -hCD3 (PE-Cy7), α -hCD4 (PE), α -hCD8 (APC) and α -hCD25 (APC-Cy7); and (2) α -hCD14 (APC), α -hCD16 (PE), α -hCD19 (APC-Cy7) and α -hCD56 (PE). The procedure was performed according to the instructions of the manufacturer. Four-color flowcytometric acquisition was performed on a FACSCanto II (BD Biosciences, San Diego, CA), using the BD FACSDiva software. A gate was set to exclude debris, and the percentages of cells expressing CD3, CD4, CD8, CD25, CD16/CD56, CD14, and CD19 were then calculated. The percentage of positive cells was corrected for values of the isotype control.

PBMC stimulation and cytokine analysis

After overnight adaptation to the culture conditions, the cells were stimulated either polyclonally using α CD3/ α CD28 antibodies (BD Biosciences; 150 and 100 ng/mL, respectively) or antigen specifically with rBet v 1.0101 (Bet v 1; Biomay, Vienna, Austria, 10 μ g/mL). Culture supernatants were harvested after 4 days of culture for α CD3/ α CD28-stimulated cells and after 1 and 7 days for Bet v 1-stimulated cells. PMA (Sigma-Aldrich, St Louis, MO, USA, 2 μ g/mL) plus Ca-I (Sigma-Aldrich, 1 μ g/mL) was added to the Bet v 1-stimulated cultures at day 6. The production of the innate and adaptive cytokines IL-1 β , IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IFN- γ and TNF- α was detected using a Cytometric Bead Array (CBA, BD Biosciences). All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen) and the procedure was performed according to the manufacturer's protocol. The detection limits according to the manufacturer were as follows: 1.1 pg/mL IL-1 β , 1.1 pg/mL IL-5, 2.3 pg/mL IL-10, 2.2 pg/mL IL-12, 0.6 pg/mL IL-13, 0.3 pg/mL IL-17, 0.3 pg/mL IFN- γ and 0.7 pg/mL TNF- α . The samples were measured on the FACSCanto II, using FCAP software (BD Biosciences).

Statistics

Because of the small group size and large number of parameters, minor differences between groups at the start of the intervention were inevitable for at least some of the parameters that were not used for randomization. Therefore, the main focus was given to intra-group variation. Data collected before treatment and after treatment were compared using the paired *t*-test. Although immune parameters may not be normally distributed, paired data were considered to be normally distributed as these are repeated measurements. Kruskal Wallis ANOVA and the Mann-Whitney *U*-test were used for comparisons between treatment groups because these data were not normally distributed. Data were considered statistically significant at the *P* < 0.05 level.

Results

In vitro screening for IL-10 production

A total of seven assays were performed with blood from PBMC from different donors. Stimulation of PBMC with candidate probiotic strains revealed a wide diversity in their capacity to induce IL-10. Whereas some strains consistently showed a strong effect (e.g. *L. plantarum* WCFS1) others repeatedly fell into the group of poor inducers of IL-10. *L. plantarum* strains were in general potent inducers of IL-10, as we have reported before [23], and therefore new strains of this species were included in the later assays. In order to combine results from these 7 independent assays, results of each assay were expressed as the relative IL-10 induction compared with the highest inducing strain found in that assay. Thereafter, for each strain, the results were averaged, and results are presented in Fig. 2. As the best IL-10-inducing strain varied between the different *in vitro* studies, and as we have averaged the results from 7 assays, we do not find a single strain that consistently had a relative IL-10 induction of one. *L. plantarum* strains looked most promising for the stimulation of IL-10. Based on these results, *L. plantarum* WCFS1, *L. plantarum* NIZO3400, *L. plantarum* NIZO2877 and *L. plantarum* CBS125632 were selected for a subsequent *in vivo* screening, although it cannot be excluded that further screening with new strains would reveal equally well or even better candidates. *L. casei* Shirota, also found to be a high IL-10 inducer, was selected as a strain for which immunomodulatory effects in allergic patients have been described previously [18]. These strains were subsequently tested in an *in vivo* study (Fig. 1).

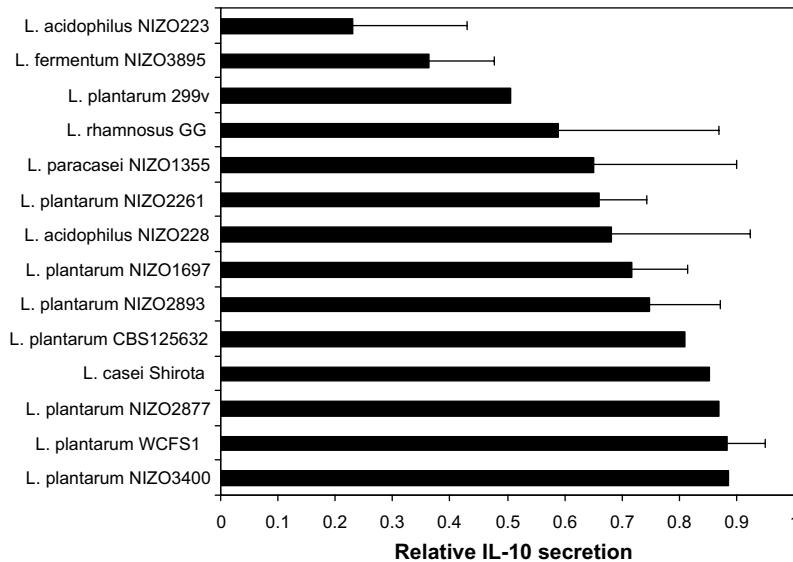


Fig. 2. Relative induction of IL-10 in a peripheral blood mononuclear cell culture assay after 24 h of stimulation with a selection of candidate probiotic bacteria. Relative values were defined as IL-10 induction by a strain compared with the highest inducing strain in the same assay. Relative data on IL-10 induction from seven assays were averaged. Bars represent average \pm standard error ($n = 1-7$).

Study population and groups

An *in vivo* screening was performed with the selected strains because the various *in vitro* studies gave variable results, and direct exposure of PBMC to candidate probiotics may not be fully predictive for immunomodulation after oral intake of the same strains. A total of 62 subjects with proven birch-pollen allergy were included in the study. Two subjects withdrew from the study for personal reasons that were not related to the study. Data from these subjects were not included in the further analysis. No subjects were excluded on basis of their blood profile; ongoing infections were not found, as was judged from the absence of high leukocyte counts, high C-reactive protein values, and/or high erythrocyte sedimentation values. In total, 60 subjects (14 men and 46 women) completed the study. As anticipated, all groups were comparable in terms of number of subjects, age, and male : female distribution (Table 1). At the start of the intervention period, serum IgE, IgG and IgG4 levels were not significantly different between placebo and any of the probiotic groups.

Table 1. Characteristics of placebo and intervention groups.

Treatment	Number of subjects	Sex (M:F)	Mean age (years)	Mean birch pollen-specific IgE (kU/L)	Mean birch pollen-specific IgG (mg/L)	Mean birch pollen-specific IgG4 (mg/L)
Placebo	10	2 : 8	36 (21-49)	27 ± 36	7.6 ± 11.2	0.7 ± 0.7
<i>L. casei</i> Shirota	10	2 : 8	35 (19-50)	17 ± 21	3.3 ± 1.7	0.4 ± 0.5
<i>L. plantarum</i> WCFS1	9	2 : 7	37 (25-48)	26 ± 30	4.8 ± 2.9	1.1 ± 1.0
<i>L. plantarum</i> NIZO3400	10	3 : 7	37 (21-46)	22 ± 28	3.5 ± 1.7	0.6 ± 0.4
<i>L. plantarum</i> NIZO2877	11	2 : 9	34 (21-47)	38 ± 50	3.7 ± 1.6	0.4 ± 0.4
<i>L. plantarum</i> CBS125632	10	3 : 7	35 (22-50)	26 ± 37	3.5 ± 1.9	0.5 ± 0.6

For age, the mean and range are presented. For antibodies, mean values ± SD are presented as determined before the intervention.

The safety of the probiotics was confirmed by clinical testing of serum parameters and by the absence of reported adverse effects during the last study visit. As anticipated, probiotic treatment did not result in increases of erythrocyte sedimentation, leukocyte counts and serum C-reactive protein concentration. The safety markers serum creatinine, bilirubin, ALT, AST and GGT were not affected in any of the treatment groups. Taken together, none of these parameters pointed to any adverse effect, which was in agreement with the absence of adverse effects of the probiotic products indicated by the subjects.

Probiotics reduce birch-pollen-specific IgE, but not IgG and IgG4

The birch-pollen-specific immunoglobulin measurements for IgE, IgG and IgG4 did not reveal statistical differences between the groups, although due to the small size of the groups, the mean values were not fully comparable (Table 1). Immunoglobulins IgE, IgG and IgG4 in the pla-

cebo group were unaffected during the 4-week study period. In contrast, birch-pollen-specific IgE levels were significantly decreased in groups receiving *L. casei* Shirota, or *L. plantarum* strains WCFS1, NIZO3400 and CBS125632. Birch-pollen-specific IgG and IgG4 levels were not affected by probiotic treatment (Fig. 3).

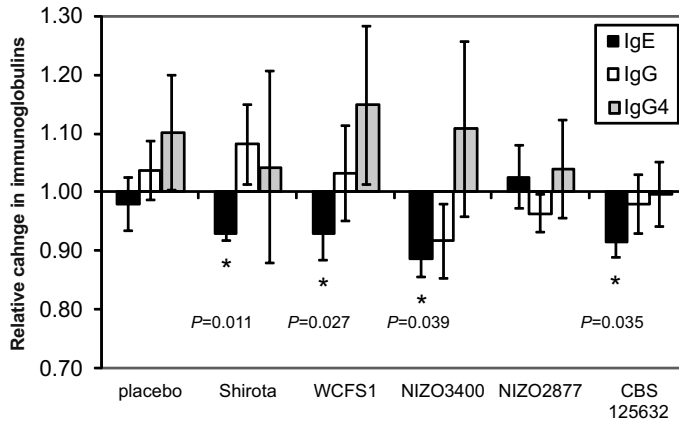


Fig. 3. Changes in immunoglobulins during the intervention period. Mean values \pm standard error are presented. P-values are presented when titers before intervention were different from titers after intervention.

***L. casei* Shirota affects PBMC cell composition by lowering CD16⁺/CD56⁺ cells**

Human PBMCs were characterized for surface markers, to evaluate whether the tested probiotics had a direct effect on the composition of blood cell populations. Large individual differences in PBMC cell composition were observed at the start of the study but all values were within the normal ranges as reported by others [29]. Before intervention, due to the relatively small group size, differences between probiotic and placebo group were observed for percentage of CD3⁺ cells (higher for the group receiving NIZO2877), percentage of CD16⁺/CD56⁺ cells (lower for NIZO2877), CD8⁺ fraction of the CD3⁺ cells (lower for WCFS1 and NIZO3400), and CD4⁺ fraction of the CD3⁺ cells (higher for WCFS1). *L. casei* Shirota induced a decrease in CD16/CD56-positive cells. No effect of probiotic intervention was seen in any of the other groups (Table 2).

***L. plantarum* CBS125632 affects cytokine production of α CD3/ α CD28-stimulated PBMC**

Human PBMCs were subjected to polyclonal or antigen-specific stimulation in order to induce cytokine production, and in this way evaluate the responsiveness of the immune system. Although cytokine production varied between individuals, stimulation with α CD3/ α CD28 antibodies for 4 days resulted in a clear cytokine production in all PBMC preparations, and detectable levels of IL-5, IL-10, IL-13, IL-17 and IFN- γ . The cytokine IL-12 was not detectable in five samples before intervention and in three samples after intervention. Probiotic treatment with *L. plantarum* CBS125632 led to a significant change in IL-5, IL-13, and IL-10 induction (Fig. 4). IL-5 was reduced by $29 \pm 11\%$ and IL-13 by $21 \pm 11\%$. IL-10 was increased by $24 \pm 13\%$. Such an effect was not seen with cells from the placebo group, or any of the other probiotic strains. The ratios between IL-10 and IL-5, indicative of the ratio between regulatory T cell activity and

Th2 activity, were significantly increased with 38% for *L. plantarum* NIZO2877, and 68% for CBS125632.

Table 2. Composition of PBMC as revealed by flowcytometry.

	Placebo	Shirota	WCFS1	NIZO3400	NIZO2877	NIZO2877
CD3+						
Before intervention	51.9 ± 2.0	57.5 ± 2.2	53.3 ± 3.0	53.7 ± 4.1	59.0 ± 2.4	56.8 ± 3.0
CD4 ⁺ /CD8 ⁺	36.7 ± 2.6	34.0 ± 3.2	29.7 ± 2.1	29.0 ± 2.3	32.5 ± 1.8	31.0 ± 1.4
CD4 ⁺ /CD8 ⁻	56.9 ± 2.8	60.1 ± 3.6	64.2 ± 2.3	64.8 ± 3.2	60.8 ± 2.3	62.7 ± 2.1
CD4 ⁻ /CD8 ⁻	4.8 ± 0.8	4.3 ± 1.1	4.6 ± 0.8	4.6 ± 1.2	5.5 ± 1.1	5.2 ± 0.9
CD4 ⁺ /CD8 ⁺	1.7 ± 0.4	1.6 ± 0.3	1.4 ± 0.2	1.7 ± 0.4	1.3 ± 0.1	1.1 ± 0.2
After intervention	51.7 ± 2.3	57.4 ± 2.2	52.5 ± 3.1	56.4 ± 2.3	63.1 ± 2.3	60.9 ± 2.3
CD4 ⁺ /CD8 ⁺	37.7 ± 2.7	33.1 ± 3.2	28.9 ± 1.4	29.8 ± 2.5	32.5 ± 1.8	31.3 ± 1.7
CD4 ⁺ /CD8 ⁻	55.5 ± 3.1	60.0 ± 3.7	64.9 ± 1.6	64.1 ± 3.6	61.4 ± 2.2	61.9 ± 2.3
CD4 ⁻ /CD8 ⁻	5.1 ± 0.8	4.4 ± 0.8	4.4 ± 0.9	4.6 ± 1.3	5.0 ± 1.0	5.5 ± 1.2
CD4 ⁺ /CD8 ⁺	1.8 ± 0.4	2.4 ± 0.7	1.8 ± 0.2	1.6 ± 0.2	1.1 ± 0.1	1.3 ± 0.2
CD25⁺						
Before intervention	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	1.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.2
After intervention	1.5 ± 0.2	2.3 ± 0.8	1.6 ± 0.3	1.3 ± 0.2	1.2 ± 0.2	1.0 ± 0.1
CD19⁺						
Before intervention	9.1 ± 1.0	9.5 ± 1.0	11.0 ± 0.9	10.2 ± 0.9	8.0 ± 0.7	9.0 ± 0.9
After intervention	9.7 ± 1.0	10.6 ± 1.0	10.0 ± 1.2	9.6 ± 1.0	8.0 ± 0.7	8.4 ± 1.0
CD16⁺/CD56⁺						
Before intervention	10.2 ± 1.4	11.3 ± 1.3	13.2 ± 2.1	9.1 ± 1.1	7.0 ± 0.9	11.2 ± 1.9
After intervention	10.0 ± 1.6	8.9 ± 1.2	13.6 ± 2.1	8.8 ± 1.1	6.8 ± 1.3	10.4 ± 1.7
CD14⁺						
Before intervention	25.6 ± 2.4	19.7 ± 1.2	19.5 ± 1.9	25.0 ± 4.2	22.7 ± 2.1	20.3 ± 2.2
After intervention	25.7 ± 2.7	20.4 ± 1.4	21.0 ± 2.3	23.5 ± 2.1	19.0 ± 1.6	18.3 ± 1.5

Mean percentages ± SEM are presented for the surface markers before and after the intervention period. Only strain *Lactobacillus casei* Shirota induced a shift in PBMC composition, by lowering the percentage of CD16⁺/CD56⁺ cells (in grey; *P* = 0.03).

To determine modulatory effects of the candidate probiotics on the proinflammatory responses, PBMCs were antigen-specifically stimulated with Bet v 1. These cells produced low but detectable levels of IL-1β, TNF-α and IL-10 within 24 h. Cytokines IL-12, IL-17 and IFN-γ were only detectable in 16, 8 and 33% of the samples, respectively. Because of the relatively high variation between samples and subjects, no effect of probiotic intervention was seen in any of the groups (data not shown).

Because of the expected low frequency of Bet v 1-specific cells in PBMC, we extended the Bet v 1-stimulated cultures to 7 days and during the last day, all viable cells were stimulated with PMA+Ca-I. The cytokines IL-5, IL-10, IL13, IL-17 and IFN-γ were detected in all supernatants. Despite the extended culture conditions, the cytokine concentrations were in general twofold to tenfold lower than from αCD3/αCD28-stimulated cells that were analysed at day 4. IL-12 was detectable in 43% of the supernatants. Because of high variation in the results from this antigen-specific assay, no statistically significant effects of probiotic intervention were seen in any of the groups (data not shown).

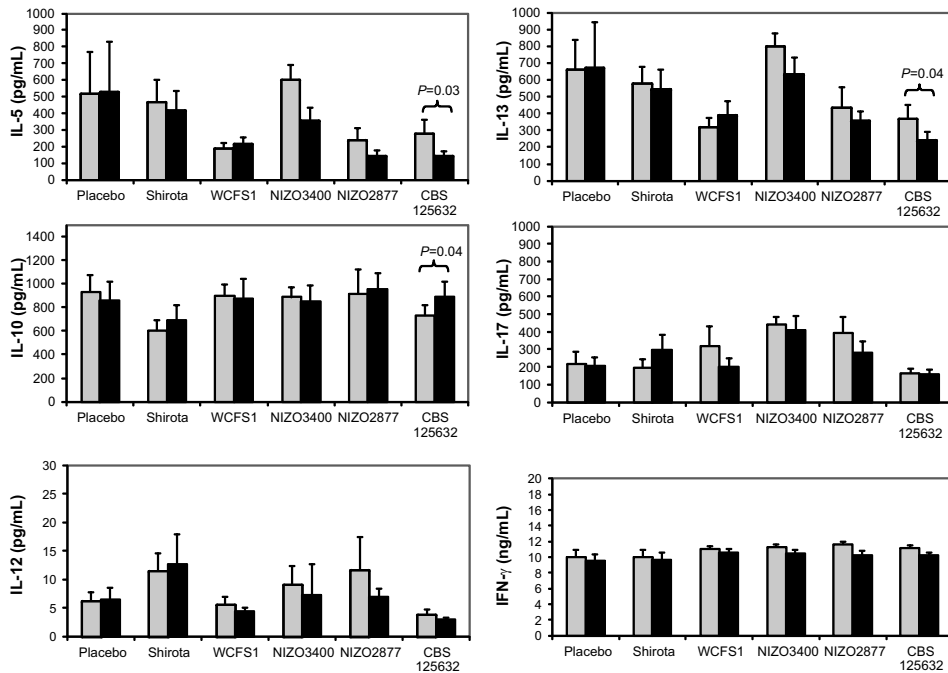


Fig. 4. Cytokines in culture medium of peripheral blood mononuclear cells stimulated with α CD3/ α CD28 antibodies after 4 days of growth. Mean values \pm standard error before intervention (grey bars), and after intervention (black bars) are represented. *P*-values indicate when measurements before and after intervention were different.

Discussion

The aim of this study was to determine whether candidate probiotic strains, selected for their *in vitro* IL-10-inducing capacity, would have a potentially beneficial immunomodulatory effect in subjects with proven birch-pollen allergy outside the hay fever season. This approach, with relatively small groups for multiple probiotics, may serve as an *in vivo* confirmation of the functionality of candidate probiotic strains that were selected on basis of *in vitro* data. The use of paired data for statistical analysis of the immune parameters highly reduces the number of subjects. However, the study does not contain clinical end-points of allergy, and therefore the true impact of the probiotics should be addressed in additional studies where end of treatment data should be compared between groups.

We observed an effect on plasma IgE levels after consumption of four candidate probiotic strains that was not seen after placebo treatment, and supporting changes in cytokine responses of stimulated PBMC after intervention with *L. plantarum* CBS125632. The candidate probiotic *L. casei* Shirota was the only strain that showed a decrease in CD16⁺/CD56⁺ cells, as well as a decline in IgE. Because safety concerns have been raised for immunomodulating probiotics [30], we included appropriate safety parameters in this study, such as infection pa-

rameters (CRP, erythrocyte sedimentation and leukocyte counts) and liver function parameters (ALT, AST, GGT and bilirubin). As anticipated, there were no probiotic-induced changes in any of these parameters in any of the included patients.

The decrease in birch-pollen-specific serum IgE that was found in four probiotic treatment groups, but not in the placebo group, was not influenced by recurrent exposure to the relevant allergens, because the study was performed during a period of the year in which antigenic stimulation with birch pollen is highly unlikely. In contrast to other antibody classes, IgE is an antibody isotype with a relatively short half life of only a few days [26]. This might explain why after this relatively short intervention period of 4 weeks we observed effects on antigen-specific IgE but not on IgG and IgG4 levels. Because the half life of IgE bound to mast cells is substantially longer than that of free serum IgE [31], the observed changes in serum do not necessarily predict changes in allergy symptoms within the time span of the study.

Allergy has long been regarded as an imbalance in Th2 and Th1 responses. Therefore, it was thought that stimulation of a Th1 response should result in a lowering of a Th2 response. However, it is suggested that Th2 responses are physiologically normal responses that can exist in the absence of allergy, and that IFN- γ secreted by Th1 cells can contribute to allergy by increasing inflammatory reactions [21], and promoting survival and functionality of eosinophils [32] and mast cells [33]. Consumption of *L. casei* Shirota led to a reduction of CD16⁺/CD56⁺ cells that was not seen for the placebo group or any of the other strains. This is a separate finding that is not backed up by other findings on this strain, and therefore we cannot fully exclude the chance finding for our observation. In contrast to our result, other studies demonstrate an increase of NK activity by this probiotic strain, either *in vitro* [34] or *in vivo* [35]. CD16⁺/CD56⁺ cells form a population of NK cells that is known for its ability to produce IFN- γ , which contributes to an increased Th1 profile. Although in our study this decrease in CD16⁺/CD56⁺ cells was not found to correlate with a decrease in IFN- γ , IL-5 or IL-13, a similar study reported a reduction of IFN- γ as well as IL-5 by *L. casei* Shirota [18]. The discrepancy between the cytokine responses in that study and our results presented here is not clear, although there are some differences between the design of the studies. One of the most striking differences is that in our study, the probiotic strains were included in a normal fermented yoghurt, which may have intrinsic immunomodulatory effects [36] or may influence the behaviour of the probiotic strain as is seen for other micro-organisms [37]. Therefore, the role of *L. casei* Shirota and the product matrix in relation to the reduction of allergy needs further investigation.

The intervention with *L. plantarum* CBS125632 led to a reduction in the production of IL-5 and IL-13 by α CD3/ α CD28-stimulated PBMC that coincided with an increase in IL-10 production. This pattern was not seen for the placebo group, and points to the direction of a promotion of T regulatory (Treg) cells that subsequently down-regulates Th2 cytokines. A role for Treg cells in the induction of tolerance has been postulated to control the development of allergy [21]. It has been reported that the number of Treg cells inversely correlates with height of the symptom scores [38]. Whether intake of *L. plantarum* CBS125632 indeed leads to reduction of allergy symptoms during the hayfever season has not been investigated and remains the subject of further studies. Although strains *L. plantarum* WCFS1 and *L. plantarum* NIZO3400 showed reduced IgE titres, the underlying mechanism to this reduction remains unclear. In addition, it would be of interest to determine whether dead probiotic cells would have a similar effect.

In this study, we have used cryopreserved cells for the cultivation of PBMC. Cryopreservation has been described to influence cell surface markers [39] and cytokine production [40]. We used in our experiments an optimized protocol for the isolation and cryopreservation of PBMC which was developed in our own laboratory as described previously [28]. We showed that no significant differences existed in the cytokine production (both in the monocyte-derived cytokines and the T cell-derived cytokines) after α CD3/ α CD28 stimulation. Therefore, we do not believe that our outcome is influenced by the use of cryopreserved cells instead of fresh cells.

We were not able to detect probiotic effects after stimulation of PBMC with the birch-pollen antigen Bet v 1 because of high variation in cytokine secretion elicited within this *ex vivo* stimulation assay. This might be explained by the longer incubation period due to the relatively low number of reactive T cells in PBMC of birch-pollen-allergic patients outside the pollen season, which are usually as low as 1 per 20 000 cells [41]. In order to monitor an effect of probiotics on this specific cell population, a pre-enrichment may be required. Nevertheless, for *L. plantarum* CBS125632, the combination of probiotic-induced changes in IgE together with supporting changes in cytokine production in α CD3/ α CD28-stimulated PBMC strongly suggests that the activity of the Bet v 1-specific sub-population of T cells is also modulated by this probiotic.

It remains speculative how oral intake of probiotic bacteria achieves the observed modulation of the immune responsiveness to allergens. It is probable that interactions between probiotic bacteria and gut mucosal tissues play a role and most likely occur in the small intestine. The gastro-intestinal tract contains the largest outer surface area of the human body, and has a high concentration of non-self molecules, such as food molecules and commensal bacteria, which are in close contact with the gut epithelium. Intake of lactic acid bacteria has an immediate effect on gene expression of the small intestinal mucosa, as has been evidenced for *L. plantarum* WCFS1 [42] and several other lactobacilli [43], but it is unclear whether this leads to systemic effects on the immune system. An alternative explanation could be that probiotic bacteria alter the microbiota composition because bacterial cell numbers and diversity of the microbiota were significantly different between allergic subjects and healthy controls, even outside the pollen season [5,6,44].

In conclusion, we present a methodology to compare candidate probiotic bacteria for their immunomodulating properties in a Th2-skewed population, as an intermediate step between *in vitro* screening and testing for clinical efficacy. Using this approach, candidate strains with a proven effect on the immune system can be selected *in vivo*. The true impact of the immunomodulation by the most effective strain, in this study *L. plantarum* CBS125632, on symptoms of birch-pollen allergy needs to be addressed in a follow-up clinical trial that targets clinical endpoints, i.e. allergic symptoms.

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Chapter *10*

General discussion

Yvonne M. Vissers

Currently there is no curative therapy available for peanut allergy, while the need for such therapy is growing both because of the severity of these allergic reactions as well as the increasing number of sufferers worldwide. Several approaches aiming at decreasing both the number of allergies as well as the severity of the food allergic reactions are now under investigation. Commonly studied therapies showing promising results are allergen-specific immunotherapy (ASIT) and anti-IgE immunotherapy (ESIT). However, for food allergy, these therapies are not yet well established and more research must provide more knowledge on the effectiveness and usefulness of these therapies. Two different approaches studied in this dissertation, are the food technological approach studied for peanuts and the immunomodulatory approach studied in birch pollen allergic patients. Our finding on these aspects will be discussed below.

Influence of processing on allergenicity of proteins

The way in which processing modifies an allergen and thereby, possibly, its allergenicity is complex and may be different from food to food or protein to protein [1]. Practical issues make it even more difficult to assess effects of processing on food allergens and, consequently, also on the whole food product. The extent of the alterations induced by processing depends on several factors, including the allergen studied (its biochemical and immunological properties), the food matrix, processing conditions applied, thermo-dynamics of allergen–IgE interaction, and patient sensitivity (threshold, tolerance, and permanency of allergenic reaction to a specific allergen) [2,3].

Mainly *in vitro* tests are used to study effects of processing as it is ethically difficult to perform *in vivo* tests in humans; however, the available *in vitro* tests all have their own drawbacks. Contradicting results between studies could be explained firstly by the use of different processing methods, performing the processing method on the whole peanut and afterwards extract the protein, or first isolating the protein and subjecting it to model thermal treatments designed to mimic the processing of the food or food ingredient itself. The impact of thermal treatment on the aggregation of proteins and hence on their (frequently resulting) insolubility, even to denaturing solvents and detergents, adds further variation to the nature of the solubilised protein, as discussed below. Secondly, differences in systems used to determine allergenic activity, such as ELISAs, Western blot or cellular assays and the clinical profile of the patients used as a source for IgE, might influence the final results.

Why processing effects are important

Humans are widely exposed to insoluble aggregated particulate forms of many allergens that might have very different properties compared to the non-aggregated soluble proteins [4]. However, with the exception of a DBPCFC, all available methods for detection and assessment of food allergens in research, clinic, and industry rely on soluble allergens, and although a SPT can be performed with the actual food, clinicians generally utilize solubilized, commercially prepared extracts for this procedure.

Even though humans mainly consume processed foods containing aggregated, insoluble fractions, studies investigating allergenic properties generally focus on soluble proteins. To study the insoluble fraction, it should first be made soluble, which will involve solubilisation

methods that could affect the structure and available epitopes of the protein. Also standardization and development of ELISA-based immunoassays is mainly based on extracts from unprocessed or raw food products [5], even though allergic individuals showed similar or higher IgE antibodies against processed food antigens as compared to raw food antigens [6]. In the recently published work of Schmitt *et al.*, the solubility of proteins in the peanut extract and protein profiles, as well as IgE-binding properties of peanut proteins in both the soluble and the insoluble fractions were assessed following different thermal processes. Overall protein solubility is reduced as a consequence of processing and IgE binding is increased in the insoluble fractions due to increased amounts of insoluble proteins, with increased time of heating in all processes tested [5]. **As a consequence, the effect of the processing methods on the structure of the protein itself and thereby its change in IgE-binding capacity could not be assessed.** In our studies described in chapters 2-6, we were mainly interested in the effect of processing on the allergenicity of the allergen itself, while correcting for the soluble protein concentration after processing. Chapter 4 showed a profound decrease in solubility of Ara h 1 after heating, which was much more pronounced for Ara h 1 than for Ara h 2/6. This difference in solubility after heating was also shown recently [5] and is most probably due to difference in aggregation behaviour. In our studies we solubilized the protein by addition of Milli-Q water and leaving the samples on a rotator for about 1 hour. We chose not to use detergents for further solubilisation as this could change the structure of the protein. Moreover, we carefully reported the protein yield after solubilisation of the treated proteins. Future experiments on the effect of processing of allergens using denaturing solvents and detergents should also take into consideration the possibility that part of the protein will eventually still stay insoluble.

To conclude, more knowledge on the influence of processing is relevant to improve the power of *in vitro* methods for diagnosing allergy, including CRD methods and cell-based assays, since most foods we eat are thermally processed and hence the original sensitising agent may be a modified, non-native, allergen or fractions thereof.

Effects of processing of Ara h 1, Ara h 2/6 and Cor a 11 on protein structure and IgE-binding and cross-linking capacity

Table 1 shows the results from our studies on effects of processing as described in chapters 2-6. The structural analysis, IgE-binding and IgE-cross-linking results are briefly discussed below.

Both the purified and the thermally treated Ara h 1 and Ara h 2/6 as well as the soluble Ara h 1 and Ara h 2/6 fractions of roasted peanuts were biochemically characterized. The analysis included SDS-PAGE, Far-UV CD, SEC, FT-IR, light scattering and AFM and these were all performed to assess both secondary and tertiary structure while FT-IR, OPA assay, anthron-sulfuric acid colorimetric microassay and NBT were performed in some of our studies to assess the extent of the Maillard reaction. Methods to assess the allergenicity of the treated allergens included IgE-binding tests, such as immunoblot, direct and indirect reversed EAST and CAP IgE measurements. However, IgE binding (*immunoreactivity*) does not necessarily reflect the potential of an allergen to also cause mediator release from basophils by cross-linking IgE on its surface (*allergenicity*). Therefore, the clinical relevance of mediator release assays using basophils is principally higher than for IgE-binding assays. IgE cross-linking was measured in our experiments by the RBL assay and the BHR test.

In chapters 2-6 and other literature [5], it was shown that proteins become less soluble with

increased time and temperature of heating, consistent with aggregation of proteins via covalent modifications other than disulfide linkages, observed as high-molecular-weight smears of the proteins in a SDS-PAGE gel. Also a low water content present during the processing, potentially enhances the Maillard reaction [5] and the propensity to aggregate and therefore also to decrease solubility. This was clearly observed from the low solubility of Ara h 1 and Cor a 11 after dry-heating in the presence of glucose described in chapters 4 and 5, respectively. Accordingly, boiling (wet-heating) compared to roasting (dry-heating) clearly decreased the rate of aggregate formation, presumably also due to the presence of water, which inhibits or decreases the extent of the Maillard reaction.

Allergen aggregation due to wet versus dry heating

After performing our wet-heating procedure of Ara h 1 described in chapter 3, the protein showed a rod-like branched aggregate structure, having a mean diameter of about 70 nm, variable heights between 10-15 nm and a Mr of > 640 kDa. Dry heating clearly resulted in hydrolysis as observed from the SDS-PAGE analysis; however, especially when heated in the presence of glucose, these degradation products assembled into very large aggregates with a diameter of 200-1000 nm. Relating these results to the effect on IgE binding and IgE cross-linking, we observed interesting differences between the wet- and the dry-heating. All treatments resulted in a decrease in IgE-binding capacity, which corresponded to a small, but significant, decrease in cross-linking capacity for the wet-heating procedures likely due to the smaller aggregates formed. Surprisingly, dry-heating resulted in a clear increase in IgE-cross-linking capacity which might be due to the very large aggregates formed of this treated protein. As the aggregates formed after wet-heating did not increase IgE cross-linking, probably the more strongly aggregated Ara h 1 fractions after dry-heating were responsible for this increase in IgE-cross-linking capacity. Interestingly, a similar result was obtained for dry-heated Cor a 11 (chapter 5), which was also shown to be strongly aggregated. We propose that large aggregates, having a high epitope density, are more efficient in cross-linking receptor bound IgE on the mast cell or the basophil surface which decreases threshold of the protein concentration to effectuate a detectable mediator release. In theory it might be possible that already one or a few large aggregates containing multiple epitopes are able to cross-link more than 100 IgE antibodies which can already induce basophil activation and subsequent degranulation [7]. In addition, epitopes present on one large aggregated protein might also cause degranulation of more than one basophil. A schematic representation of this effect is shown below.

Smaller aggregates exhibit both a reduced IgE binding capacity in fluid phase and a reduced IgE cross-linking capacity

Dimerization of allergens can be important for *in vitro* [8] and *in vivo* IgE-cross-linking potential as seen for the Bet v 1 allergen in mice [9]. The Bet v 1 molecule is rather small (17 kDa) also in comparison with IgE antibodies (188 kDa) and moreover, IgE is rather rigid through the absence of a hinge region, and its flexibility is limited when bound to FcεRI, where IgE is fixed in a bent shape [10,11]. **This could make cross-linking by this small allergen difficult from a stereochemical point of view.** The distance between adjacent FcεRI-bound IgE molecules is between 8-24 nm to make a stable contact with the protein, therefore small molecules might have difficulty to cross-link two IgE molecules [7]. However, in our experiments, we showed that the native

monomeric Ara h 2/6, having a comparable Mr (15-19 kDa) was able to cross-link IgE, possibly due to different amounts and different spatial arrangements of IgE-epitopes on Ara h 2/6 versus Bet v 1, as well as a possible difference in percentage of allergen-specific IgE to total IgE in the sera used in these studies. As a consequence, cross-linking may already have been effective for monomeric Ara h 2/6, but not for monomeric Bet v 1. In addition, dimerization or oligomerization of Ara h 2/6 occurring after processing further decreased IgE-cross-linking capacity, possibly because epitopes were less well accessible to the cell-bound IgE antibodies. This would corroborate a recently proposed model explaining the hypoallergenic activity of a Bet v 1 trimer (Fig. 1).

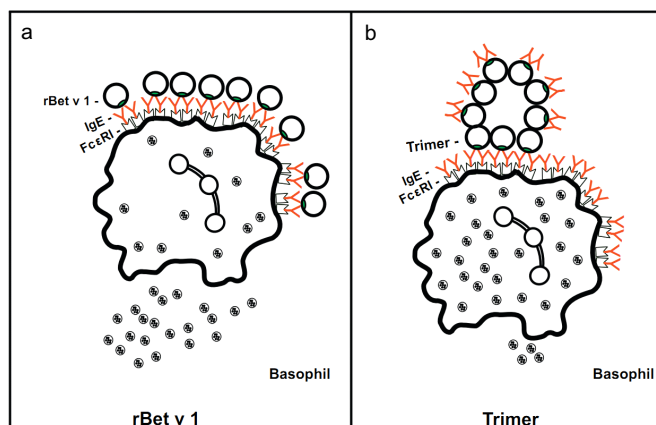


Fig. 1. Model for hypoallergenic behaviour of rBet v 1 trimer. Monomeric Bet v 1 or the trimeric Bet v 1 can bind a comparable number of IgE antibodies in solution. Monomeric molecules (a) may be more efficient in cross-linking of effector cell-bound IgE than the trimer (b) as only a portion of the IgE epitopes on the aggregated trimers comes into an optimal position for cross-linking of effector cell-bound IgE (Adapted from [12]).

A recombinant Bet v 1 trimer was shown to exhibit a strongly reduced ability to induce basophil activation, showed a stronger IgE reactivity in a solid phase immunoassay, but a reduced allergenic activity in fluid phase IgE experiments. The Bet v 1 trimer occurred in fluid phase in the form of high molecular weight aggregates (>600 kDa) of which only a certain percentage of the IgE epitopes available on the surface of this molecule formed a spatial arrangement that allows cross-linking of cell-bound IgE. A considerable number of the IgE epitopes is less well accessible to cell-bound IgE antibodies, which could explain the decrease in cross-linking capacity of this trimer (Fig. 1 [12]). The spatial arrangement of IgE epitopes on a given protein may thus have an important influence on the allergenic activity of this protein in cross-linking effector cell-bound IgE.

Differential IgE cross-linking capacity for processed Ara h 1

This model (Fig. 1) might also explain the decrease in cross-linking capacity of the 'small' aggregated structures observed for Ara h 1 after wet-heating applied in our study. An extension of this model is proposed in Fig. 2. Native Ara h 1 (Fig. 2A), present as a trimer, is able to cross-link IgE more effectively (cross-linking more FcεR1 per surface area) compared to the aggregated structure obtained after wet-heating in the presence or absence of glucose (Fig. 2B). In addition, our processing procedures might also have caused destruction of IgE epitopes and epitope masking because of the oligomerisation which could all contribute to the decrease in IgE binding and IgE cross-linking. Dry-heating in the absence of glucose resulted in hydrolysis

products or possibly monomers of Ara h 1. The resulting increase in the number of fragments of different sizes leads to an enhanced ability to cross-link more receptor bound IgE molecules. Part of the degradation products were assembled into much larger aggregate structures, having a high epitope density and therefore one aggregate might cross-link more receptor-bound IgE molecules at once (Fig. 2C). The very large Ara h 1 aggregates formed upon dry-roasting in the presence of glucose contain a high epitope density and one aggregate can therefore cross-link several IgE molecules at once which could cause a rapid degranulation of the cell (Fig. 2D). In addition, when a few epitopes are bound to the antibody, other receptors, having the flexibility to migrate in the membrane, also bind to the epitopes present on the aggregate (binding cooperatively) resulting in dense spots of bound IgE molecules on the membrane of the basophil all cross-linking a large number of FcεR1.

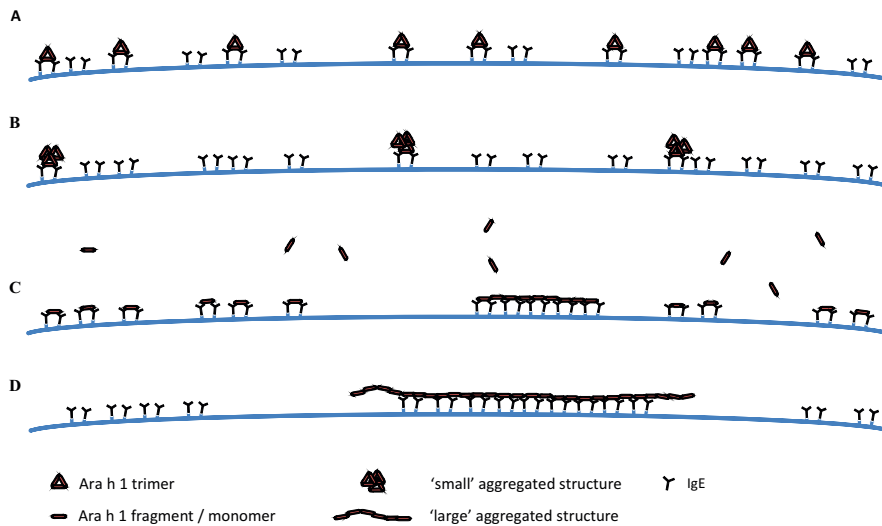


Fig. 2. Model for differential behaviour of Ara h 1 before and after processing for IgE cross-linking in the mediator release assay. (A) Native Ara h 1 (B) wet-heated Ara h 1 heated in the presence or absence of glucose for 15 min at 100°C (C-D) dry-heated Ara h 1 heated in the absence (C) or presence (D) of glucose for 20 min at 145°C.

Differential IgE binding capacity for processed Ara h 1

For IgE binding in the EAST inhibition assays, all treated Ara h 1 allergens showed a decrease in IgE binding capacity. Also here, interesting differences were observed between the different treatments which might be explained by the model shown in Fig. 3. Overall, larger structures will be sterically hindered and therefore less able to reach the IgE which is bound to the anti-IgE fixed on the plate. The tracer molecules (N-Ara h 1) are much smaller and are more easily able to reach and bind to the IgE molecules. The decrease in IgE-binding capacity is therefore most pronounced for Ara h 1 dry-heated in the presence of glucose (Fig. 3D). Ara h 1 dry-heated in the absence of glucose showed only a minor decrease in IgE-binding capacity, as more small Ara h 1 hydrolysate products and possible Ara h 1 monomers are present which still contain

intact epitopes and can easily bind to the IgE. The few large aggregates present will be less able to bind to the IgE (Fig. 3C).

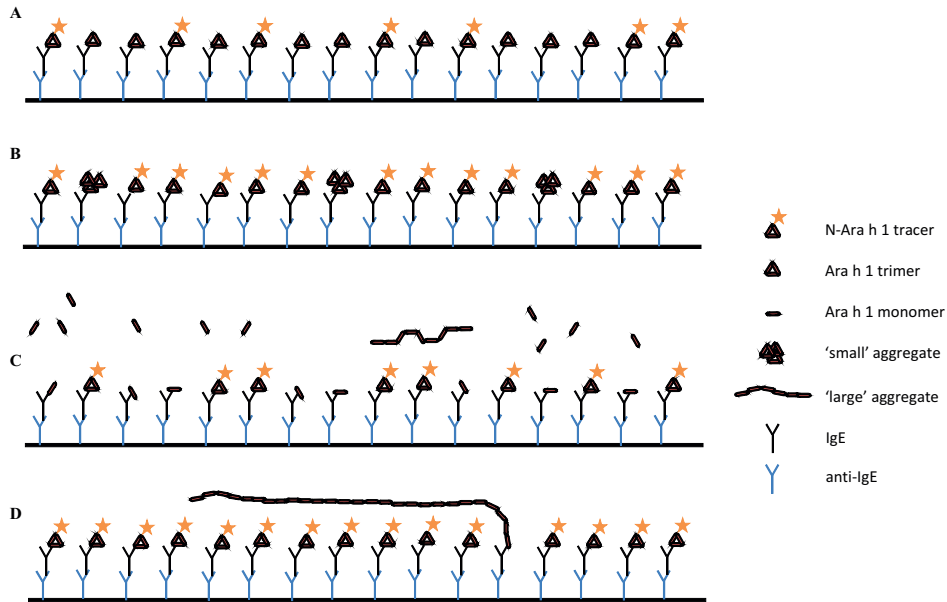


Fig. 3. Model for differential behaviour of Ara h 1 before and after processing for IgE binding in the EAST inhibition assay. (A) Native Ara h 1 (B) wet-heated Ara h 1 heated in the presence or absence of glucose for 15 min at 100°C (C-D) dry-heated Ara h 1 heated in the absence (C) or presence (D) of glucose for 20 min at 145°C.

Maillard reaction

A more extensive Maillard reaction occurring during dry-heating of both Ara h 1 and Ara h 2/6 in the presence of glucose, seems to partly counteract the increase and respectively decrease in IgE cross-linking capacity (Table 1). A protective effect of glucose was also observed for dry-heated Ara h 2/6 but not for dry-heated Ara h 1, with respect to IgE binding. In addition, for wet-heated Ara h 2/6, both MRAs indicated a protective effect of the presence of glucose during heating. However, for IgE cross-linking of Ara h 1 and for both IgE binding of Ara h 1 and Ara h 2/6 the presence of glucose during wet-heating even slightly further decreased the IgE-cross-linking and IgE-binding capacity. Therefore, the effect of Maillard reaction does not seem to be consistent, which was also shown in the literature mentioned in the introduction section. However, a protective effect of glycation has also been observed after applying a severe heat-treatment of the LTP from apple (Mal d 3) which resulted in a significant decrease in allergenicity and a protective effect of glycation [3].

Ara h 1 isolated from roasted peanuts lost much of its native structure, was highly denatured and showed no evidence of glycation but showed a similar IgE-binding capacity as native Ara h 1. It was previously demonstrated that new IgE binding sites might be created by Maillard reactions on peanut allergens [13,14]; however, as Ara h 1 isolated from roasted peanuts

was not as extensively glycosylated as the model processed G-Ara h 1 which showed a reduction in IgE binding and cross-linking capacity, this suggests that other modifications than the Maillard reaction contributed to its allergenicity. The question remains if the fraction isolated from the roasted peanuts resembles the protein when it is still in the peanut itself.

Ara h 2/6 (chapter 2) clearly shows different properties than Ara h 1 (chapter 3) with respect to the wet-heating procedure. The wet-heating procedure also resulted in a clear decrease in IgE-binding capacity, as measured with the reverse EAST inhibition, which corresponded with a decrease in cross-linking capacity, measured using both the RBL-assay as well as the BHR-test (Table 1). **In contrast to Ara h 1, the decrease in reactivity after heating seemed more pronounced in the MRAs than in the IgE-binding assay.**

Table 1: Overview of results of the effect of processing compared to literature (as indicated by the arrows)

Protein	Dry/ wet Heat ing	Ch.	Structure		Tertiary (LS) (d.nm)	(AFM) (h.nm)	MR	IgE binding		IgE cross-linking	
			Secondary (Mr in kDa)	Ch.				Reverse inhibition	RBL	BHR	
Ara h 1	-	N	3/4	Native like (β-sheet rich)	16	4 (native, trimeric)	n				
	Wet	H	3	Formation of intermolecular β-sheets & aggregation (more in H than in G)	85	10-15 (rod-like branched aggregate)	n	↓ (715)	↓ (6.9)		
		G	3		91	10-15 (simpler rod-like structure)	y	↓ (1236)	↓ (7.5)		
	Dry	H	4		mainly 12, also 30-400 200-1000		y	↓ (3.6)	↑ (1700)		↑
		G	4				y	↓ (9000)	↑ (700)		↑
		R	3	Random coil (β-sheet ↑, α-helix ↓, some intermolecular β-sheets)	50	7-8 (more globular, shorter rods)	n	≈ as native			
Conclusion											
								(N=R>DH>WH >WG>DG)	(dH>dG>N>G>H)	(dH=dG>N)	
Ara h 2/6	-	N	2/4	α-helix rich, monomers (16)			n				
	Wet	H	2	Random coil; mainly dimers (28-30); 20% monomers (16), oligomers (44-150), hydrolysis products (<16)				↓ (9.6)	↓ (150)		↓
		G	2					↓ (14.5)	↓ (130)		↓ (<wH) ^a
	Dry	H	4					↓ (600)	↓ (661)		↓
		G	4					↓ (22)	↓ (19)		↓ (<dH) ^a
		R	2	α-helix rich (like N); mainly monomeric (like N)				≈ as native (slightly ↓)			
Conclusion											
								(N≥R>WH>WG>DG>DH)	(N>dG>WG>WH>H)	(N>WG>WH) (N>dG>DH)	

N: native; H/G: heated in the absence or presence of glucose, respectively (heating at 100°C or 110°C for 15 min for wet-heating of Ara h 1 and Ara h 2/6, respectively; heating at 145°C for 20 min for dry-heating of both Ara h 1 and Ara h 2/6); R: fraction of protein isolated from roasted peanuts; WPPE: whole peanut protein extract; Ch.: Chapter; LS: light scattering; d: diameter; AFM: atomic force microscopy; h: height; MR: Maillard reaction; n: no; y: yes; ↓: decrease; ↑: increase; ↑: wet-heating; d:dry-heating; ^a: glycation reduced IgE cross-linking less compared to heating in the absence of glucose

Clinical relevance of IgE cross-linking and IgE binding affinity

Differences in the amount of the allergen required to degranulate FcεR1 cells could give an indication of the clinical relevance of the allergen (e.g. 1-10 µg/ml, most probably non-clinically cross-reactive protein, 0.001-10 ng/ml for major purified class 1 allergens) [15-21]. This was also observed from the RBL assay performed in chapter 5, in which we showed that a 50% release was obtained around 2 µg/ml using peanut sera containing cross-reactive IgE to the hazelnut protein tested, Cor a 11. Sera high in Cor a 11 specific IgE showed a 50% release at lower concentrations of ~0.2 µg/ml, which were even lower after dry-heating (~1 ng/ml). The rather low sequence similarity (33-34%) of Cor a 11 and Ara h 1 compared to other 7S globulins [22], could yield fewer, low-affinity allergen-IgE antibody reactions [23]. The allergen-specific IgE antibodies concentration, affinity, clonality (epitope-specificity) and the ratio of allergen-specific IgE to total IgE are all shown to impact on the extent of effector cell activation, and e.g. a higher-affinity IgE binding to Der p 2 was associated with higher levels of basophil activation [15,24]. Moreover, individual IgE antibodies are able to bind single allergenic epitopes present in the different isoallergens with a broad range of affinities. This further contributes to the complexity of IgE-allergen interactions at the effector cell surface [16].

Recently it was also found that IgE against unheated and heated products could be functionally different (specificity, clonality, affinity) and therefore could differentiate allergic from sensitized patients. By using a competitive peptide microarray assay, it was observed that IgE binding from unheated-milk and baked-milk tolerant individuals was primarily of low affinity, while milk allergic individuals had IgE that bound certain epitopes of milk proteins with high affinity. Therefore, besides a greater IgE epitope diversity, a higher affinity could also be associated with clinical phenotypes and severity of milk allergy [25,26]. This difference in IgE affinity, will most probably also be important in cross-reactive IgE, as discussed above. Table 2 gives an overview of the different IgE binding and degranulation tests and the linear and or conformational epitopes that the test recognizes, including remarks on affinity of the antibody binding.

Table 2. Overview of IgE binding and degranulation tests.

Method	Epitopes recognized (L or both L & C)	Measuring IgE binding/ cross-linking	Remarks on affinity ^a
Specific IgE (RAST/CAP/ direct ELISA)	Linear	IgE binding	Detects also low-affinity IgE Ab
SPT	Both	IgE cross-linking (by mast cell activation in skin)	
Immunoblotting	Linear	IgE binding	Detects also low-affinity IgE Ab
Reverse EAST inhibition	Both	IgE binding	Affinity is important
Mediator release assays	Both	IgE cross-linking	Quantity of allergen needed for release might indicate allergenicity

L: linear; C: conformational; ^a Range of affinity of IgE K_d is 10^{-10} – 10^{-12} M. Sensitivity of Der p 2-induced histamine release from human basophils was closely related to the IgE-antibody affinity of Der p 2. IgE-antibody affinity for Der p 2 ranged from 0.056 to 1.77×10^{-10} M [24].

Linear and conformational epitopes

A recent meta-analysis was performed on allergy-related data from the immune epitope database and revealed over 4,500 allergy-related epitopes derived from 270 different allergens covering about 17% of known allergens. The majority of epitopes were defined for B cells/antibodies and IgE-mediated reactivity, and relatively fewer T-cell epitopes, mostly related to CD4+/MHC class II. Interestingly, the majority of food allergen epitopes were B-cell epitopes [27]. The epitopes reported represent unique molecular structures experimentally shown to react with a B-cell or T-cell receptor. The structures are generally categorized as minimal/optimal epitopes (11–15 amino acid residues), larger less well-defined regions (20–50 residues), and key residues identified as being involved in binding (1–2 residues for T cells and 4–5 amino acid residues for IgE binding). The majority of known epitopes on allergens comprise linear epitopes. To identify linear epitopes on allergens, epitope mapping with synthetic peptides has been demonstrated to be a suitable tool. IgE-binding abilities are subsequently evaluated by a method based on ELISA using patient sera [28,29]. However, conformational IgE epitopes generally represent the main IgE-binding structural motif on allergens. It has been postulated that reactivity and thus specific IgE antibodies to conformational epitopes of foods, mainly egg or milk, are more associated with development of tolerance, while reactivity to linear epitopes are more associated with food allergy persistence. Children who outgrew their milk allergy showed milk-specific IgE antibodies primarily directed against conformational epitopes while those with persistent milk allergy also showed IgE antibodies directed against specific sequential epitopes [30,31].

Short peptides representing the main IgE-binding regions of Ara h 2 were used in competition experiments (both ELISA and RBL cell assay) and showed little contribution to the IgE binding nor to the cross-linking of the allergen. Therefore, sequential epitopes, even though they can readily be identified by direct IgE binding to short peptides, contribute little to the IgE binding of Ara h 2, particularly in fluid phase antibody/target interaction. Assuming that fluid phase binding of IgE antibodies is more relevant in relation to *in vivo* allergenicity, it is possible that solid phase approaches for epitope identification may generate partially misleading results. Similar results were obtained with unfolded rAra h 2, which indicates a predominance of IgE recognition of conformational epitopes on Ara h 2 and minor involvement of sequential epitopes [32]. **However, conformational epitopes are in general more susceptible to destruction** by physical and chemical treatments as observed for the conformational epitope in Ana o 2, the 11S globulin from cashew [33,34]. Currently, there is still a debate about the relevance of conformational epitopes in peanut allergens and food allergens in general.

So far, not many potentially relevant IgE-binding regions of food allergens, particularly conformational epitopes formed by sequentially discontinuous amino acid residues, have been described. The dominance of protruding areas and the presence of a region with alternating sign in the electrostatic potential are compatible with favourable antigen–antibody interactions. It has been proposed that strong electrostatic interactions should be expected in high-affinity antibody–antigen complexes, whereas weaker electrostatic activity inherent to less specific contacts should be indicative of cross-reactivity. Most of antibody epitopes are described to be conformational and hence, sequential approaches may render limited information, besides might exhibit reduced antibody-affinity [35,36]. **Many of the structural requirements for binding of epitopes to antibodies were resolved for IgG antibodies which have a preference for pro-**

truding loops and secondary structures in antigens. IgE antibodies, however, recognise different binding sites than IgG antibodies as was described for α -lactalbumin [37] and with crystal structures of β -lactoglobulin, Phl p 2, Api m 2, Bet v 1 and Bla g 2 [38,39]. So far, however, only a few sequences of allergen-specific IgE antibodies have been characterized that are complementary to the known allergenic epitopes. Thus, further studies on the exact nature of B-cell activating and IgE-binding epitopes on allergens is necessary to elucidate further functional studies on allergenicity of (food derived) allergens.

Conclusions and future research

Depending on the test system, heating of allergens may have no effect or it may decrease or increase their immunoreactivity (IgE binding capacity) and/or allergenicity (basophil degranulation capacity). The existence of sequential and conformational epitopes, the demasking of new epitopes or the modification of existing epitopes can explain effects conferred by different processing methods. In addition, protein oligomerization and aggregation, as a result of processing, was found in our studies to be an important factor in how processing alters allergenicity of proteins. Moreover, physiologically relevant *in vitro* models of digestion testing the ability of these large aggregated fragments to survive digestion, could indicate possible differences in digestibility between the native and the aggregated allergens, which might contribute to the overall allergenicity of the protein. Aggregated structures might be more resistant to digestion and could reach the intestinal lumen in an undigested form and thereby protect the epitopes from digestive enzymes. In addition, it is essential to relate changes in IgE binding and in IgE-cross-linking capacity to changes in the minimum eliciting dose in a DBPCFC and therefore of relevance in establishing a proper diagnosis of the individual allergic patient. As an example it has been observed that a 100-fold reduction in IgE binding increased the minimum eliciting dose by only 2-fold [40]. Therefore, to establish thresholds for processed foods, would still require the use of the DBPCFC.

Even though the boiling method tested for Ara h 1 and Ara h 2/6 might only partially decrease the allergenicity of these peanut proteins and thereby possibly the allergenicity of the whole peanut, other methods are also under investigation. Recently physical processing by irradiation has been described to promote polypeptide fragmentation and hydrophobic surface modification, leading to protein misfolding, and subsequently, to aggregation. Effects on stability of food allergens after gamma irradiation might contribute to the development of methods to reduce allergenicity [41], however the allergenicity of these aggregates formed should be tested in IgE binding and IgE cross-linking assays. Complete elimination of the allergenic potential of foods by using processing methods will be unlikely given the current knowledge on processing and allergenicity. The allergenic potential of a food protein can be divided into its capacity to sensitize susceptible individuals resulting in the production of specific IgE antibodies and its ability to elicit an allergic reaction in already sensitized patients. In the current thesis the allergenic potential of a food protein to elicit an allergic reaction was studied. Ara h 1 and Ara h 2/6 are relevant major allergens, however studying these allergens is not sufficient to predict the effect on whole peanuts.

Studying the IgE-binding reactivity and the cross-linking capacity of the insoluble and thus more heavily aggregated proteins will provide insight into the role that these physico-chemical modifications play in the overall allergenic reaction cascade. It will be a challenge to purify and

test these strongly aggregated structures. In addition to test our hypothesis, dry-heating can be applied to more allergens to assess tertiary structure and thereby the extent of aggregate formation from fragmented polypeptides. Afterwards these processed allergens could be tested for their allergenic potential in both IgE-binding and IgE-cross-linking assays. Better characterization of interactions with other proteins, fat, and carbohydrates in the food matrix will be important for future studies on the effect of processing on a whole food product.

Immunomodulation by lactic acid bacteria

In the introduction section several approaches for allergy management are described that are currently under investigation. One of these approaches is the use of lactic acid bacteria as potential probiotics. Clinical studies support that lactic acid bacteria such as lactobacilli may represent a promising preventive and therapeutic strategy for allergic diseases. However, the scientific proof of the effect of lactic acid bacteria in allergic patients has been controversial as some studies do not seem to prove a beneficial effect. Modulation of the immune system by lactic acid bacteria is presumed to be an important mode of action and was the main focus of the studies on lactic acid bacteria performed in this thesis.

Comparison of results of both *in vitro* and *in vivo* studies of different research groups is complicated because each study used different study conditions. The choice of the probiotic species/strains used, growth phase of the bacteria, dosing and time of exposure, use of live or dead bacteria, the patient populations studied and the read-out system and markers used could all influence the outcome and conclusions of the study. In addition, for *in vivo* studies host factors such as current immune status and age, and also standardization of components in the diet that may bias the effect of the lactic acid bacteria and food matrix composition and processing effects should be taken into account when designing the protocol [42]. The design of the study is therefore of utmost importance as this will enhance the likelihood of finding significant effects and/or finding clinical benefit of the tested lactic acid bacteria.

In vitro studies

Even though the final proof of principle is to perform a well-designed double-blind placebo controlled *in vivo* trial, *in vitro* studies are indispensable to properly design such a study. The number of claims and effects attributed to lactic acid bacteria are increasing tremendously. New scientific requirements for proving solid evidence of health claims of probiotics related to gut and immune function are urgently needed as more and more health claims of probiotics are currently being rejected by the European Food Safety Authority (EFSA). However, substantial research on probiotics shows clear strain-specific immunomodulatory effects [43-48], which is also confirmed by our studies described in chapter 7-9. Thus it is essential to reliably select the most promising candidates for desired health applications.

Large numbers of candidate strains are often tested for immunomodulating properties in a variety of *in vitro* models to select those strains with the best characteristics according to single immune parameters, like IL-10, IFN- γ or IL-12 inductive capacity [49-53]. Based on the study presented in chapter 7, in which initially 51 *Lactobacillus* strains were tested, bacterial species could be classified for their probiotic characteristics on the basis of their immune profiles.

Generally, *L. plantarum* strains were high IL-12 inducers whereas strains from *L. acidophilus* were recognized as low IL-12 inducers. As we were specifically interested in a possible benefit of these strains in allergic patients, we hypothesized that *L. plantarum* strains were better candidates as they might be better in skewing T-cells differentiation toward a Th1-like response. To test this hypothesis, immune cells were cultured of patients with a proven pollen allergy, who are known to have Th2-skewed allergic immune responses with high specific IgE levels in the serum, and selected strains were tested for their immunomodulatory properties in chapter 8. Differences were observed between the strains in their capacity to induce IFN- γ , IL-12 and TNF- α production and interestingly, all strains were able to suppress IL-13 induction, one of the hallmark cytokines in allergy. PBMC derived from healthy patients are most often used to investigate possible probiotic effects. However as immunomodulatory effects could exert a differential effect in patients having different diseases reflected by a different immune status, we chose patients with seasonal allergy as the target group for *in vitro* testing (chapter 8). Our results show that PBMC of patients with seasonal allergy were appropriate to determine immunomodulatory activities of probiotic bacteria *in vitro*.

***In vivo* studies**

A further selection of lactic acid bacteria was applied *in vivo* in birch-pollen-allergic subjects. Often the selection of probiotic strains is based on *in vitro* screening only, in several cases supported by validation in animal models. However, only few studies compare *in vitro* to *in vivo* data and a good correlation was found in some studies [54-56], but not in all [57]. Clinical trials are often performed with only a single probiotic strain, which bears the risk that not the most appropriate strain is used if the selection was based on *in vitro* studies only. Therefore, elucidation of probiotic functionality should preferably be based on *in vivo* validation of multiple strains, to validate the proposed immunomodulatory capacities observed *in vitro*. Based on results from chapter 7 and 8 and on an additional screening on IL-10 inducing properties, five *Lactobacillus* strains were selected for an *in vivo* study, including 62 birch-pollen allergic subjects who consumed yoghurts for 4 weeks. The human *in vivo* screening system used in chapter 9, was found appropriate to evaluate selected immunomodulatory strains prior to testing for clinical efficacy.

From our *in vitro* studies, *Lactobacillus plantarum* appeared the most promising strains for potential use in allergic patients and in our *in vivo* study *L. plantarum* CBS125632 showed both a decrease in birch-pollen-specific IgE as well as a decrease in Th2 cytokines by anti-CD3/anti-CD28-stimulated PBMC and an increase in IL-10 production. This strain might confer its effect by stimulating Treg cells and would thus be a good candidate for a follow-up clinical trial that targets clinical endpoints of allergy.

Lactic acid bacteria and allergy

There is insufficient but very promising evidence to recommend the addition of probiotics to foods for prevention and treatment of allergic diseases. Several recent reviews discuss the current evidence for beneficial effects of probiotics in allergy and overall, most success has been obtained for prevention of atopic dermatitis [42,58-65]. Clinical improvement especially in allergic rhinitis and IgE-sensitized (atopic) eczema has been reported too, but these studies are less conclusive. Data for food allergy/hypersensitivity and allergic airway diseases are less

adequate at this moment. Heterogeneity between studies preclude evaluation of efficacy of probiotics and might explain the differential effects with respect to the outcome measures observed between studies.

Several randomized controlled trials (RCTs) have been performed to study the role of probiotics as a treatment modality in allergic rhinitis. Probiotic intake generally improved quality of life score in patients with allergic rhinitis and decreased the number of episodes of rhinitis per year. Adverse events were not recorded and probiotic therapy might thus be useful in rhinitis, but the present data do not yet allow any treatment recommendations [62].

Moreover, probiotic bacteria engineered to deliver IL-10 or IL-12 might be able to decrease food-induced anaphylaxis and provide a proof of principle and a putative treatment option to prevent IgE-type sensitization to food allergens [66,67].

Mechanism of action of lactic acid bacteria

Lactic acid bacteria are known to influence both mucosal and systemic innate and adaptive immune function. Of particular importance is their potential to elicit antigen-specific secretory IgA responses at mucosal surfaces and their intrinsic adjuvant properties to induce strain-dependent pro-inflammatory and regulatory cytokines *in vitro* and *in vivo*. Different probiotic strains result in differential cytokine production in human PBMC and it has been shown that there were strain-specific differences in the production of IL-12 and IFN- γ , but that all bacteria tested did induce TNF- α [50-53]. **Probiotics can exert a plethora of immune-mediated functions** as described by Turpin *et al.*, including their responsive factors. These immunostimulatory factors include bacterial DNA, lipoteichoic acid, MAMPs, secreted factors such as H₂O₂, passive and active binding and indirect increase in butyrate levels [52].

Bacterial cell wall components are in direct contact with several immune cell types by binding various PRRs of the host. The variation in composition and quantity of cell-wall structures such as the polysaccharide-peptidoglycan complex, teichoic acid composition and extent of glycosylation between lactobacilli might partly explain differential immunomodulation properties of different strains. For example, recent studies on DNA from Lactobacilli showed anti-inflammatory effects of natural commensal-origin DNA on the gut which was mediated via TLR9 signalling [68,69] and provided the basis for the rational design of a TLR-9 vaccine based on a Amb a 1 - CpG DNA construct for ragweed allergic individuals. Vaccination activates TLR-9 in dendritic cells inducing activation of intracellular signalling pathways leading the immune response away from a pro-allergic response with Th2 cytokines (IL-4 and IL-5). In addition, the vaccine reduces the ability of the Amb a 1 allergen to bind and cross-link IgE bound to mast cells. As is clear from many *in vitro* studies, dendritic cells play a key role in regulating T-cell responses and lactic acid bacteria have been shown to differentially modulate dendritic cells maturation in a strain-dependent manner [51]. Activation of human dendritic cells by *Lactobacilli* was shown to skew T-cell responses toward Th1 and Tc1 pathways [70]. Currently, there is a lot of interest into Treg inducing capacities of probiotics [50,71,72]. As we observed in both our *in vitro* and *in vivo* studies an increase in IL-10 production, that coincided with a reduction in the production of IL-5 and IL-13, these specific lactic acid strains tested in our studies might also have been responsible for promotion of Treg cells that subsequently downregulated Th2 cytokines, although this has not been elucidated on the level of individual cytokine producing CD4+ T-cells.

Apart from TLR, lactic acid bacterial antigens can be also recognized through molecules like nucleotide-binding oligomerization domain receptors (NODs) or lectins, including the C-type lectin dendritic cells specific intra-cellular adhesion molecule 3-grabbing nonintegrin (dendritic cells-SIGN) and even the mannose receptor CD206 on the gut immune cells in mice [73-75]. However, additional work is needed to delineate how different lactic acid strains could exert their effect. This could give more inside on the use of specific strains for targeted application in specific diseases. As also faecal transplantation becomes part of mainstream medicine [76], studies on interactions of humans with their microbiota will allow better insight into their synergistic functionality.

Essential characteristics of lactic acid bacteria

The debate is still on-going whether probiotics should be alive to exert their effect, as beneficial responses can both be generated by both live and dead cells in probiotic products. Live probiotic cells can influence both the gastrointestinal microbiota and the immune response whilst the (cell-wall) components of dead cells exert a, possibly TLR-mediated, immunomodulating response in the gastrointestinal tract and beyond. There is no evidence that probiotic supplements are able to replace the existing microbiota when these have been killed off and bacterial levels in faeces disappear within days when supplementation ceases. Modification of the microbiota in the gut could be useful in the treatment of clinical conditions affecting the gastrointestinal tract while potential benefits extend beyond the gut by modulation of the immune system possibly beneficial for inflammatory diseases, allergy, respiratory diseases and others [77]. **Comparable studies between live and dead cells showed conflicting results [78-80].** However, dependent on the mechanism of action of the probiotic strain in the target group tested, live bacteria could exert a more pronounced effect. Especially if colonization of probiotics to the wall of the gut is thought to exert the beneficial effect, e.g. resulting in reduction of the virulence of the pathogens by competition for binding sites or direct killing of the pathogenic bacteria, live bacteria will be a better choice. Several studies show immunomodulating effects by inactivated bacteria [81-85] which was also shown from our study described in chapters 7 and 8. In addition, both live cells surviving the GI-tract, dead cells, and their metabolites can contribute to the effects in the GI-tract as these are all present in probiotic preparations. With respect to the commercial use of probiotics, dead cells would be desirable as they are easier to use in a great variety of products. They are considerably safer for the host, the shelf life is no longer a restriction and standardization in their formulation is easier than for live preparations [77,86,87]. Especially the standardization of probiotic formulations can be a very important factor because the culture method of the bacteria and potentially even the growth stage of bacteria in a preparation can induce differential responses in humans [88]. In our *in vitro* studies we used heat-killed bacteria, to compare different strains and to enable the analysis of possible immunomodulating effects of lactic acid bacteria on the T-cell level, which requires extended culture periods.

To conclude, even though the definition of probiotics specifically states that the bacteria should be alive, immunomodulating effects are also observed by inactivated bacteria like in our studies described in chapters 7 and 8. Several important advantages of the use of dead cells, could make them a better choice to use in a probiotic product.

Lactic acid bacteria in the diet: the future

The potential to influence the immunological response via immunomodulatory food ingredients such as probiotics, has shown to have potential not only from the perspective of allergy prevention, but also from a more general health related perspective. It has been clearly demonstrated that diet, including therapy by probiotics has a considerable effect on the composition of the gut microbiota and immune responses [89-91]. Studies on the therapeutic use of probiotic formulations in irritable bowel syndrome, Crohn's disease, *Helicobacter pylori* therapy, cancer induction, allergy, skin and urogenital diseases as well as administration to ease post-operative complications are on-going. Recently, it has even been shown that adult germ-free mice displayed a different behaviour from mice with normal microbiota, suggesting that during a critical period early in life microorganisms affect the brain and change the behaviour later in life [92]. Although the conclusions from the different studies are still controversial with respect to the beneficial effects of probiotic supplementation on allergy development, these studies provide a valuable lesson for the therapeutic use of lactic acid bacteria. Careful strain selection and characterization of the immunomodulatory properties will be essential prior to application in prophylactic and therapeutic settings.

Rather than developing new anti-inflammatory drugs, carefully designed trials to establish whether diet directly affects inflammatory disease as well as through what cellular and molecular mechanisms, could be more cost-effective [90]. **Sequencing of genomes and transcriptomes** that can be correlated to measured effects and thereby enable testing which bacterial genes and derived components are essential to specific immunomodulatory properties could reveal more about the mechanisms [50,51,93-95]. Development of probiotics from microbiota derived from human communities in which allergies and asthma are almost completely non-existent could also be an approach which is worthwhile to investigate further. In addition, as the underlying immunological mechanisms of allergies might be similar for patients having seasonal allergies and patients having food allergy, it will be interesting to test strains which have been shown to have a beneficial effect in patients having seasonal allergies (e.g. possibly strain *L. plantarum* CBS125632 tested in our studies) also for their immunomodulating effects in food allergic patients.

Conclusions and future research

That specific lactic acid bacteria confer a beneficial effect in specific patients with diverse diseases and that an array of mechanisms could be responsible for the different observed effects has become clear over the last years. This opens opportunities for specific and differential use of lactic acid bacteria in allergic individuals. However, future well-designed clinical trials, testing a diverse array of strains and involving large numbers of a diverse array of diseased patients, will be mandatory to achieve definite evidence of the preventive and curative role of lactic acid bacteria in medical practice and to finally develop proper advice and guidelines [61]. Therefore, also with respect to allergy, the use of probiotics should be further investigated before this could be recommended as a common treatment of allergic patients.

In addition, there is a need to invest greater efforts to properly select candidate probiotic strains to be included in clinical trials and translation of *in vitro* models to *in vivo* models for different diseases will be crucial for this. Small-scale human trials, like the methodology presented in chapter 9 used to compare candidate probiotic bacteria for their immunomodulating

properties in a Th2-skewed population, could be an appropriate choice to select candidate strains for testing in clinical trials.

Concluding remarks

The described studies which are part from the EuroPrevall project, showed that the model boiling procedure applied on the purified allergens, resulted for both Ara h 1 and Ara h 2/6 in a decrease in IgE-binding capacity as well as in IgE-cross-linking capacity. This is likely caused by the smaller aggregates formed after boiling. In contrast, dry-heating of Ara h 1 but not Ara h 2/6 resulted in formation of very large aggregates composed of fragmented polypeptides containing a high epitope density which are likely responsible for the decrease in IgE-binding capacity and the increase in IgE-cross-linking capacity observed. Moreover, our data demonstrated that using only IgE-binding assays is insufficient to fully assess allergenicity of a modified allergen and that effects on the biological activity, such as mediator release, must also be considered.

As we showed that processing procedures have a profound effect on the allergenicity of the allergen, we consider our results of importance in studies of the allergenic potential of food proteins and for the proper diagnostic allergy testing of the individual patient using both CRD and DBPCFC. For diagnostic purposes purity of a protein is often checked by SDS PAGE however, aggregation could also play an important role. Moreover, standardization of the allergenic products used in DBPCFC, will be important to facilitate comparison of test results between allergology centres and can also be important to improve the test procedure. In addition, including these considerations could be effective to improve the current risk assessment of allergenic proteins, however no general rules can be formulated at this moment.

In the second part of this thesis, strain-specific immunomodulatory effects were observed of the lactic acid bacteria tested. This stresses the importance of a proper pre-selection of candidate strains which would enable to choose the most promising strains for clinical testing. Overall, the lactic acid bacteria tested in our studies were capable to modulate PBMC of healthy and allergic patients to induce innate cytokines, decrease synthesis of the Th2 cytokine IL-13, increase synthesis of the regulatory cytokine IL-10 and differentially induce IFN- γ and IL-12 production. *Lactobacilli* might confer a beneficial effect in allergic patients based on these immunomodulatory effects. Five strains were selected for a small-scale human trial and four strains showed a decrease in birch-pollen specific IgE. Additionally, one strain also decreased Th2 cytokine induction and a follow-up clinical trial should prove the clinical efficacy of this strain for probiotic use in patients having a seasonal allergy.

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Summaries and acknowledgements

Summary
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Acknowledgements

Summary

Allergic diseases such as allergic rhinitis, allergic asthma, atopic eczema and food allergy have become an increasing health problem world-wide, affecting between 20-30% of the total population. Peanut allergy (prevalence ~1%) is a common and persistent food allergy accounting for severe allergic reactions. Peanuts are often consumed after thermal processing (e.g. boiling, roasting) which can alter the protein structure and change its immunoreactivity and allergenicity. *In vitro* diagnostic testing, however, is generally performed using the native, unprocessed protein and more knowledge on the effect of processing on allergens is necessary to improve these diagnostic procedures. In addition, rationally designed processing could also lead to reduction of the allergen content in certain products and therefore be an effective food technological approach in allergy management. Another approach in allergy management is the use of immunomodulating foods, such as probiotics. There are indications that probiotics, e.g. specific lactic acid bacteria, could be beneficial for many conditions, including different clinical expressions of allergy.

Chapter 1 gives an overview of several aspects of allergy with a focus on food allergy. Firstly the basic mechanism and the involved immune cells are discussed, after which the prevalence of food allergy in the context of the EuroPrevall project is described. Different food allergens are discussed with an emphasis on the allergens from peanut and different methods are described to assess the potential allergenicity of proteins under widely used processing conditions, including heating and the Maillard reaction. Lastly, different methods to prevent or treat allergies are discussed with a special emphasis on immunomodulation by lactic acid bacteria. This introduction chapter is concluded with the research aim and thesis outline.

Section 1: Influence of processing on allergenicity of proteins

In our first study, described in **Chapter 2**, Ara h 2/6 was purified from raw peanut and heated in solution (boiling) in the presence or absence of glucose. Ara h 2 and 6 were also purified from roasted peanuts for comparison. Structural changes, the capacity to induce cell proliferation and cytokine production, and IgE-binding and IgE cross-linking capacity were evaluated. Although no effect of processing on T-cell reactivity was observed, heat-induced denaturation reduced the IgE-binding and cross-linking capacity. Interestingly, the soluble fraction of the Ara h 2/6 isolated from roasted peanuts retained the conformation and allergenic activity of the native protein.

In **Chapter 3** similar methods were used to assess the effect of heating and glycation on Ara h 1. Heating in solution, irrespective of their level of glycation, resulted in formation of aggregates having reduced IgE-binding and cross-linking capacity, while T-cell reactivity was retained. The soluble fraction of Ara h 1 isolated from roasted peanuts appeared to be highly denatured, formed more globular and smaller aggregates, and showed no evidence of glycation. However, these smaller aggregates retained IgE-binding capacity, unlike the aggregates formed after heating and glycating purified Ara h 1. These results could account for observed differences between boiled and roasted peanuts and suggest that other modifications than the Maillard reaction affect the allergenicity of Ara h 1.

As peanuts are often consumed after roasting, the wet-thermal processing procedures, em-

ployed in the two previous described studies, were related to the effect of thermal treatment and Maillard reaction under low moisture conditions, which is described in **Chapter 4**. The extensive heating at low moisture resulted in hydrolysis of both Ara h 1 and Ara h 2/6. However, in contrast to Ara h 2/6, soluble Ara h 1 formed large aggregates. Thermally treated Ara h 2/6 had both a lower IgE-binding and degranulation capacity compared to the native form, and the presence of glucose during heating partly counteracted both the decrease in IgE-binding and degranulation capacity. The IgE-binding capacity of Ara h 1 was also decreased; however, the basophil degranulation capacity increased significantly. This demonstrates the importance of including degranulation assays in addition to IgE-binding assays, when assessing allergenic potency of allergens. In addition, we here propose a role for large aggregates in the increased IgE-cross-linking capacity of individual allergens.

Chapter 5 describes the effect of glycation on the immunoreactivity and basophil degranulation capacity of Cor a 11, the 7S globulin from hazelnut (and thus a homologue of Ara h 1). Three processing methods (heating at low moisture content at 37, 60 and 145°C) resulted in proteins with increasing degrees of glycation. Glycation at 37°C did not influence the specific IgG or IgE binding, while both were decreased after heating at 60°C and 145°C. However, heating at 145°C in the absence or presence of glucose resulting in the formation of aggregated structures, increased the basophil degranulation capacity of Cor a 11 using sera high in Cor a 11 specific IgE, but not when using sera from peanut allergic patients low in Cor a 11 specific IgE. Therefore, this study besides showing the importance of the use of a combination of tests also indicated the importance of using well-characterized sera as a source of IgE.

In **Chapter 6** we focused on the clinical features of all our clinically well-defined peanut allergic patients of which immune cells and sera were used for the previously described studies. In addition, soy allergic patients were included and an extensive IgE profile was determined for all patients. Gly m 4 (Bet v 1 homologue from soy) sensitization was suggested to be an important indicator of severe soy allergy in the soy allergic patients, while in peanut allergic patients sensitization to allergens from soy and pea extract nor Gly m 5 and 6 was found to have a good diagnostic specificity. This is likely due to the presence of clinically non-relevant cross-reactivity between peanut-specific IgE and homologues soy and pea components.

Section 2: Immunomodulation by *Lactobacillus* strains

In the first *in vitro* study, described in **Chapter 7**, initially 51 *Lactobacillus* strains were screened of which 8 were selected and tested for their immunomodulating effects on peripheral blood mononuclear cells (PBMC) of healthy donors. All tested *Lactobacillus* strains were capable of inducing the production of IL-1 β , IL-10, IFN- γ and TNF- α . Clear strain-specific effects were observed with *L. plantarum* strains showing significantly higher induction capacity of IFN- γ , IL-12 and TNF- α compared with *L. acidophilus* strains. We therefore concluded that especially *L. plantarum* strains are promising candidates in IgE-mediated allergy by their stimulation potential of the T-cell response toward a putative Th1 response.

As healthy subjects, in contrast to allergic individuals, are assumed to finely regulate the Th1/Th2 balance by inducing sufficient Treg cell activity, immunomodulatory effects of six selected *Lactobacillus* strains were investigated on PBMC of pollen-allergic patients in **Chapter 8**. All strains could modulate PBMC to induce innate cytokine production and in addition, all strains had the ability to repress IL-13 production. Again a differential effect on IFN- γ and IL-12

induction was observed. In addition, one strain could extensively suppress proliferation induced by anti-CD3/anti-CD28 stimulation. Specific strains that were able to suppress the Th2 cytokine induction and induce Th1 cytokines might be beneficial for allergic patients.

Effects found *in vitro* cannot directly be extrapolated to *in vivo* and therefore, in **Chapter 9**, we performed an *in vivo* screening including five *Lactobacillus* strains. Blood samples were collected before and after a 4-week intervention with probiotics from all 62 birch-pollen-allergic patients included. Four strains caused a decrease in birch-pollen-specific IgE and for one specific strain this coincided with significant decreases in IL-5 and IL-13 and an increase in IL-10 production by anti-CD3/anti-CD28 stimulated PBMC cultures and might therefore have the potential to alleviate seasonal allergy symptoms.

The last chapter, **Chapter 10**, gives an overview of the most important results of this thesis and discusses the research limitations and future research perspectives. We hypothesize the role of protein aggregation in allergenicity and we elaborate on the importance of a proper stepwise approach to realize selection of a proper lactic acid strain for *in vivo* human testing.

In conclusion, this thesis showed that processing effects can have profound and specific effects on the structure and the allergenicity of relevant allergens. However, to test putative effects on allergenicity, IgE-binding tests only are not sufficient and mediator release assays are important to include, particularly when testing aggregated proteins. These results might have consequences for the proper diagnosis of food allergy in daily practice. Finally, as effects of lactic acid bacteria are strain specific, a proper pre-selection of candidate strains is important to choose the most promising strains for clinical testing. In our *in vivo* screening, one strain, *L. plantarum* CBS125632, was found to be promising because of its desired immunomodulatory activity to test in a follow-up trial to reduce symptoms of birch-pollen allergy.

Samenvatting

Allergische aandoeningen zoals allergische rhinitis, allergische astma, atopisch eczeem en voedselallergie zijn wereldwijd een steeds belangrijker gezondheidsprobleem geworden, waaraan 20-30% van de totale bevolking lijdt. Pinda-allergie is een veel voorkomende (~1% van de bevolking) en persistente voedselallergie, verantwoordelijk voor ernstige allergische reacties. Pinda's worden vaak gegeten na een bepaalde thermische behandeling (bijv. koken of roosteren) en deze behandeling kan de eiwitstructuur en daarbij de immunoreactiviteit en allergeniciteit veranderen. Diagnostische *in vitro* testen worden echter meestal uitgevoerd met native, onbehandelde eiwitten en meer kennis over het effect van de behandeling van allergenen is nodig om deze diagnostische testen te verbeteren. Daarnaast kunnen specifieke behandelmethode ook leiden tot een afname van de hoeveelheid allergeen in een product. Deze methoden kunnen daardoor een effectieve voedingstechnologische aanpak zijn voor het onder controle houden van allergie. Een andere aanpak van allergiemanagement is het gebruik van immunomodulerende voedingsproducten zoals probiotica. Er zijn indicaties dat probiotica, bijvoorbeeld melkzuurbacteriën, heilzaam zijn voor diverse lichamelijke condities, o.a. bij diverse allergische symptomen.

Hoofdstuk 1 geeft een overzicht van diverse allergische aspecten, met een focus op voedselallergie. Eerst worden het basale mechanisme en de diverse betrokken immuuncellen bediscussieerd, waarna de prevalentie van voedselallergieën in de context van het EuroPrevall project wordt beschreven. Verschillende voedselallergenen, met de nadruk op allergenen uit de pinda, en verschillende methoden om de allergeniciteit van eiwitten te bepalen worden besproken. Tot slot worden diverse methoden aangehaald om allergieën te voorkomen en te behandelen, met de nadruk op immunomodulatie door melkzuurbacteriën. Deze introductie wordt afgesloten met het onderzoeksdoel en een inhoudelijk overzicht van het proefschrift.

Sectie 1: Invloed van behandeling op de allergeniciteit van eiwitten

In de eerste studie, beschreven in **hoofdstuk 2**, werd Ara h 2/6 gezuiverd uit pinda's die verhit werden in een waterige omgeving (koken), in de aan- of afwezigheid van glucose. Ara h 2 en 6 werden ter vergelijking ook gezuiverd uit geroosterde pinda's. Veranderingen in de eiwitstructuur, de capaciteit om celdeling te bewerkstelligen, cytokine productie te induceren, IgE-antistoffen te binden en IgE-antistoffen te crosslinken werden geëvalueerd. Hoewel het koken geen effect had op de T-cel reactiviteit, was de IgE-binding en IgE-crosslinking capaciteit van het door hitte gedenatureerde eiwit verminderd. Tevens was het opmerkelijk dat de oplosbare fractie van Ara h 2/6 dat uit geroosterde pinda gezuiverd werd, de conformatie en de allergeniciteit van het native eiwit wel behield.

In **hoofdstuk 3** werden vergelijkbare methoden gebruikt om het effect van verhitten en niet-enzymatische glycosylering op een ander pinda-allergeen, Ara h 1, te bepalen. Onafhankelijk van de mate van glycosylering, resulteerde verhitting van Ara h 1 in een waterige oplossing in de vorming van aggregaten met een verminderde IgE-binding en IgE-crosslinking capaciteit, terwijl de T-cel reactiviteit werd behouden. De oplosbare fractie van Ara h 1 uit geroosterde pinda's bleek in grote mate te zijn gedenatureerd, vormde rondere en kleinere aggregaten, en was niet geglycosyleerd. Deze kleinere aggregaten behielden echter wel de IgE-

bindende capaciteit, in tegenstelling tot de aggregaten die gevormd werden na verhitting en glycosylering van geïsoleerd Ara h 1. Deze resultaten zouden een verklaring kunnen zijn voor de waargenomen verschillen tussen gekookte en geroosterde pinda's en suggereren dat andere veranderingen dan de Maillardreactie de allergeniciteit van Ara h 1 beïnvloeden.

Aangezien pinda's vaak geroosterd worden geconsumeerd, zijn de behandelmethoden van de allergenen in oplossing beschreven in de vorige twee studies opgevolgd door een studie naar het effect van een hitte-behandeling en de Maillardreactie onder lage vochtigheid, zoals beschreven in **hoofdstuk 4**. De intensieve verhitting bij lage vochtigheid resulteerde in hydrolyse van zowel Ara h 1 als Ara h 2/6. In tegenstelling tot Ara h 2/6 vormde Ara h 1 echter grote aggregaten. Hitte-behandeld Ara h 2/6 vertoonde een verlaagde IgE-binding en degranulatiecapaciteit vergeleken met de natieve vorm. De aanwezigheid van glucose tijdens de verhitting werkte zowel de afname in IgE-binding als de afname in degranulatiecapaciteit tegen. De IgE-bindingscapaciteit van Ara h 1 werd ook verminderd na verhitten, maar de capaciteit om basofielen te degranuleren werd significant verhoogd. Dit laat zien dat het zeer belangrijk is om een degranulatietest naast de IgE-bindingstest mee te nemen bij het bepalen van de allergische potentie van allergenen. Bovendien stellen we voor dat de grote aggregaten een rol spelen bij de toename van de IgE-crosslinking capaciteit van individuele allergenen.

Hoofdstuk 5 beschrijft het effect van glycosylering op de immuunreactiviteit, gemeten als basofieldegranulatiecapaciteit, van Cor a 11, de 7S-globuline uit hazelnoot (een homoloog van Ara h 1). Drie behandelmethoden (verhitten bij lage vochtigheid en 37, 60 of 145 °C), resulteerden in eiwitten met een verhoogd glycosylatieniveau bij de oplopende temperaturen. Glycosylering bij 37 °C had geen invloed op de specifieke IgG- of IgE-binding, terwijl beide waren afgenomen bij 60 en 145 °C. Verhitting bij 145 °C in aan- of afwezigheid van glucose resulteerde echter in de vorming van aggregaten en vergrootte de basofieldegranulatiecapaciteit van Cor a 11 getest met sera met een hoog gehalte van Cor a 11-specifiek IgE. Bij gebruik van pinda-allergische patiëntensera met een laag gehalte van Cor a 11-specifiek IgE werd echter geen verschil in degranulatiecapaciteit waargenomen. Deze studie laat, naast het naar voren brengen van het belang van het gebruik van een combinatie van testen, ook het belang van het gebruik van goed gekarakteriseerde sera als bron van IgE zien.

In **hoofdstuk 6** richtten we onze aandacht op de klinische kenmerken van al onze klinisch goed gedefinieerde pinda-allergische patiënten van wie de immuuncellen en sera werden gebruikt in de hiervoor beschreven studies. Daarnaast zijn er soja-allergische patiënten aan de studie toegevoegd en werd van alle patiënten een uitgebreid IgE-profiel bepaald. In dit hoofdstuk wordt voorgesteld dat Gly m 4 (een Bet v 1 homoloog eiwit uit soja)-sensibilisatie een belangrijke indicator is voor ernstige soja-allergie in soja-allergische patiënten, terwijl in pinda-allergische patiënten sensibilisatie voor allergenen uit een soja- of erwtexttract, net als Gly m 5 en 6, een slechte diagnostische voorspellende waarde bleken te hebben. Dit kwam waarschijnlijk door de aanwezigheid van klinisch niet-relevante kruisreactiviteit tussen pinda-specifiek IgE en homologe soja en erwt componenten.

Sectie 2: Immunomodulatie door *Lactobacillus* stammen

In de eerste *in vitro* studie, beschreven in **hoofdstuk 7**, werden 51 *Lactobacillus* stammen gescreend, waarvan er 8 werden geselecteerd en getest op hun immuunmodulerende effecten op perifere mononucleaire cellen uit bloed (PBMC) van gezonde donoren. Alle

geteste *Lactobacillus* stammen induceerden de productie van IL-1 β , IL-10, IFN- γ en TNF- α . Duidelijke stam-specifieke effecten zoals een significant hogere productie van IFN- γ , IL-12 en TNF- α werden geobserveerd wanneer de *L. plantarum* stammen werden vergeleken met *L. acidophilus* stammen. Hieruit concludeerden we dat voornamelijk *L. plantarum* stammen veelbelovende kandidaat-stammen zijn in IgE-gemedieerde allergieën door hun mogelijkheid om de T-cel reactie in de richting van een potentiële Th1 reactie te stimuleren.

In **hoofdstuk 8** werden de immuunmodulerende effecten van zes geselecteerde *Lactobacillus* stammen onderzocht op PBMCs van pollen-allergische patiënten. Alle stammen waren in staat om de 'innate' cytokineproductie door PBMCs te stimuleren en daarnaast waren alle stammen ook in staat om de IL-13-productie te onderdrukken. Ook hier was een stam-specifiek effect waarneembaar qua IFN- γ - en IL-12-inductie. Eén stam was in staat om anti-CD3/anti-CD28 geïnduceerde celdeling te onderdrukken. De specifieke stammen die de Th2-cytokineproductie verminderden, en daarnaast Th1-cytokines versterkten, zouden mogelijk heilzaam kunnen zijn voor allergische patiënten.

Effecten die verkregen zijn met *in vitro* testen kunnen niet direct worden vertaald naar de *in vivo* situatie, en daarom hebben we in **hoofdstuk 9** een *in vivo* screening uitgevoerd met vijf *Lactobacillus*-stammen. Bloedmonsters van 62 berkenpollen-allergische patiënten werden zowel voor als na een 4-weken durende probiotica-interventie verzameld. Interventie met 4 van de 5 stammen zorgde voor een afname in berkenpollen-specifiek IgE en voor één specifieke stam ging dit samen met een significante afname in IL-5 en IL-13 en een toename in IL-10 productie in anti-CD3/anti-CD28 gestimuleerde PBMC-kweken. Deze stam zou hierdoor mogelijk geschikt kunnen zijn om seizoensgebonden allergische symptomen te verlichten.

In het laatste hoofdstuk, **hoofdstuk 10**, wordt een overzicht gegeven van de belangrijkste resultaten uit dit proefschrift en worden de beperkingen van de uitgevoerde studies en toekomstige onderzoeksmogelijkheden besproken. We bespreken de mogelijke rol van eiwitaggregatie in relatie tot de allergeniciteit en we wijden uit over het belang van een goede stapsgewijze aanpak om tot een selectie van melkzuurbacteriën te komen voor *in vivo*-testen.

Uit dit proefschrift kan worden geconcludeerd dat behandeling van eiwitten belangrijke en eiwit-specifieke effecten kan hebben op zowel de structuur als de allergeniciteit van relevante allergenen. Om de allergeniciteit van eiwitten te kunnen testen is het echter niet voldoende om alleen IgE-bindingstesten te gebruiken maar ook basofielhistaminedegranulatietesten zijn hierbij van groot belang, in het bijzonder als men geaggregeerde eiwitten wil testen. Deze resultaten kunnen consequenties hebben voor het stellen van een goede diagnose van voedselallergie in de dagelijkse praktijk.

Gezien de aangetoonde stam-specifieke effecten van melkzuurbacteriën, is een goede voorselectie van potentiële stammen van groot belang alvorens de meest belovende stammen te kiezen voor een klinische studie. In onze *in vivo* screening werd *L. plantarum* CBS125632 gezien als de meest belovende stam vanwege de gewenste immuunmodulerende effecten van deze stam. Deze stam zou daarom getest kunnen worden in een follow-up trial voor symptoomvermindering bij patiënten die lijden aan berkenpollenallergie.

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Promoveren kan niet zonder ontspanning, sport en afleiding: Anne, Ellen, Karin, Marieke en Yvonne, ik hoop dat we altijd vriendinnen zullen blijven. Onze uitstapjes naar Venetië en België waren fantastisch en onze borrels en etentjes zijn altijd weer op-en-top gezellig! Ik hoop dan ook echt dat we dit altijd zullen blijven doen!

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About the Author

List of publications

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List of publications

Publications in international journals

Vissers YM, Jansen APH, Ruinemans-Koerts J, Wichers HJ, Savelkoul HFJ: IgE component-resolved allergen profile and clinical symptoms in soy and peanut allergic patients. *Allergy* **2011** *in press*

Snel J, **Vissers YM**, Smit BA, Jongen JMJ, van der Meulen ET, Zwijsen R, Ruinemans-Koerts J, Jansen APH, Kleerebezem M, Savelkoul HFJ: Strain-specific immunomodulatory effects of *Lactobacillus plantarum* strains on birch-pollen-allergic subjects out of season. *Clin Exp Allergy* **2011** (41), pp. 232-242

Vissers YM, Snel J, Zuurendonk PF, Kleerebezem M, Wichers HJ, Savelkoul HFJ: *Lactobacillus* strains differentially modulate cytokine production by hPBMC from pollen-allergic patients. *FEMS Immunol Med Microbiol* **2011** (61), pp. 28-40

Vissers YM, Snel J, Zuurendonk PF, Smit BA, Wichers HJ, Savelkoul HFJ: Differential effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* strains on cytokine induction in human peripheral blood mononuclear cells. *FEMS Immunol Med Microbiol* **2010** (59), pp. 60-70

Mandalari G, Adel-Patient K, Barkholt V, Baro C, Bennett L, Bublin M, Gaier S, Graser G, Ladics GS, Mierzejewska D, Vassilopoulou E, **Vissers YM**, Zuidmeer L, Rigby NM, Salt LJ, Defernez M, Mulholland F, Mackie AR, Wickham MSJ, Mills ENC: *In Vitro* Digestibility of β -Casein and β -Lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. *Regulatory Tox. and Pharm.* **2009** (55), pp. 372-381

Jeurink PV, **Vissers YM**, Rappard B, Savelkoul HFJ: T-cell responses in fresh and cryopreserved peripheral blood mononuclear cells: kinetics of cell viability, cellular subsets, proliferation and cytokine production. *Cryobiology* **2008** (57), pp. 91-103

Mandimika T, Baykus H, **Vissers Y**, Jeurink PV, Poortman J, Garza C, Kuiper H, Peijnenburg A: Differential gene expression in intestinal epithelial cells induced by single and mixtures of potato glycoalkaloids. *J. Agric. Food. Chem* **2007** (55), pp. 10055-66

Submitted manuscripts

Vissers YM[#], Blanc F[#], Stahl Skov P, Johnson PE, Rigby NM, Przybylski-Nicaise L, Bernard H, Wal J-M, Ballmer-Weber B, Zuidmeer-Jongejan L, Szépfalusi Z, Ruinemans-Koerts J, Jansen APH, Savelkoul HFJ, Wichers HJ, Mackie AR, Mills ENC, Adel-Patient K; Effect of heating and glycation on the allergenicity of 2S albumins (Ara h 2/6) from peanut. (*submitted*)

Vissers YM, Iwan M, Adel-Patient K, Stahl Skov P, Rigby NM, Johnson PE, Mandrup Müller P, Przybylski-Nicaise L, Schaap M, Ruinemans-Koerts J, Jansen APH, Mills ENC, Savelkoul HFJ, Wichers HJ; Effect of roasting on the allergenicity of major peanut allergens Ara h 1 and Ara h 2/6: the necessity of degranulation assays. (*submitted*)

Blanc F[#], **Vissers YM[#]**, Adel-Patient K, Rigby NM, Mackie AR, Gunning PA, Wellner NK, Stahl Skov P, Przybylski-Nicaise L, Ballmer-Weber B, Zuidmeer-Jongejan L, Szépfalusi Z, Ruinemans-Koerts J, Jansen APH, Bernard H, Savelkoul HFJ, Wichers HJ, Mills ENC; Boiling peanut Ara h 1 results in formation of aggregates with reduced allergenicity. (*submitted*)

Iwan M[#], **Vissers YM[#]**, Fiedorowicz E, Kostyra H, Kostyra E, Savelkoul HFJ, Wichers HJ; The impact of Maillard reaction on immunoreactivity and allergenicity of the hazelnut allergen Cor a 11. (*submitted*)

[#] Equal contribution

Side publications

Book chapter

Chapter 12: Influence of food processing, digestion and the food matrix on allergenicity & cellular measures of allergenicity. **Vissers YM**, Wichers HJ and Savelkoul HFJ; In: Multidisciplinary Approaches to Allergies, Edited by Zhongshan Gao, Huahao Shen, Min Zheng, Lynn J. Frewer and Luud J.W.J.Gilissen, Co-publishing by Zhejiang University Publishing and Springer, **2011** (*in press*)

Article in Dutch allergy journal

Vissers Y, Wichers HJ, Savelkoul HFJ; Voedseleiwitten en allergische reacties. Ned Tijdschr Allergie **2007**(7), pp. 160-165

Overview of completed training activities

Name of the course	Graduate school/institute	Year
Discipline specific activities		
<i>Courses</i>		
Industrial proteins	VLAG	2006
Ecophysiology of the gastrointestinal tract	VLAG	2007
Medical immunology	Avans Hogeschool	2007
<i>Conferences and meetings</i>		
Allergy Consortium Wageningen PhD day ‡	ACW	2006
NVvI course	NVvI	2007
Allergy School Bischoffsheim	EAACI	2007
Gut Day	WUR/TNO/VLAG	2007
WIAS Science day	WIAS	2007
Minisymposium on food allergy	Wenckebach Inst (UMCG)	2008
Allergy and research ACW	Studium Generale WUR	2008
Meeting Dutch Research School Allergology †	DRSA	2008
Minisymposium food allergy; defence M.A. Bollen	WUR	2009
Allergy School Norwich †	EAACI / GA ² LEN	2009
Genetics and immunology of IBH in horses	WIAS	2009
NVvA meeting	NVvA	2010
Fish immunology workshop	WIAS	2010
Utrecht allergy symposium	UAC	2010
EAACI Congress, Londen †	EAACI	2010
EMF symposium	WIAS	2010
Symposium advances in biology of APCs and T cells	VUMC	2010
Symposium immunology; defence C.M.S. Ribeiro	WIAS	2010
Congress FAAM, Venice ‡	EAACI	2011
Conferences ‡ (Doorwerth, Madrid, Warsaw, Berlin, Vienna, Florence)	EuroPrevall	06/10
General courses/activities		
Organising and supervising thesis work	DO	2006
VLAG PhD week	VLAG	2007
PhD competence test	WGS	2007
Project and time management	WGS	2007
Scientific writing	Language Centre WUR	2008
BD FACS Diva workshop	BD	2008
Mini symposium Bibliometrics	WUR library	2009
BD Multicolor workshop	BD	2009
NWO Talent Days	NWO	09/10

Optional courses and activities

Preparation of PhD research proposal		2006
PhD study trip United Kingdom, France		2006
Organization of PhD study trip China		2008
PhD study trip China †		2008
Meetings in the group		06/11
Visit Institute of Food Research (IFR), Norwich, UK	IFR	2009
Visit Paul-Ehrlich-Institute (PEI), Langen, Germany	PEI	2009
Visit INRA, Jouy-en-Josas, France	INRA	2009

† Poster presentation

‡ Oral presentation

Abbreviations

ACW	Allergy Consortium Wageningen
APCs	Antigen presenting cells
BD	Becton, Dickinson and Company
DO	Educational Staff Development Group
DRSA	Dutch Research School Allergology
EAACI	European Academy of Allergy and Clinical Immunology
EMF	Electromagnetic field
FAAM	Food Allergy and Anaphylaxis Meeting
GA ² LEN	Global Allergy and Asthma European Network
IBH	Insect bite hypersensitivity
IFR	Institute of Food Research
INRA	Institut National de la Recherche Agronomique
NVvA	Dutch Society for allergology
NVvI	Dutch Society for immunology
NWO	Dutch Organisation for Scientific Research
PEI	Paul-Ehrlich-Institute
UAC	Utrecht Allergy Consortium
UMCG	University Medical Center Groningen
VUMC	VU university medical center
VLAG	Graduate School for Nutrition, Food Technology, Agrobiotechnology, and Health Sciences
WGS	Wageningen Graduate Schools
WIAS	Wageningen Institute of Animal Sciences
WUR	Wageningen University and Research Centre

Curriculum vitae

Yvonne Maria Vissers was born in Veghel on 8th of July 1982. In 2000, after graduating from the Kruisher College in Uden (vwo), she started her study in Human Nutrition and Health at the Wageningen University. She chose for a free master program in which she completed several projects. A minor thesis at the department of Toxicology discussed the problem of combination toxicology. It investigated the influence of different flavonoids on the transport of an important heterocyclic amine in the human diet using Caco-2 cells as a model. During her major thesis performed at the Cell Biology and Immunology department she optimized the cryopreservation method of peripheral blood mononuclear cells and performed allergen specific stimulation cultures. Yvonne spent the last 6 months of her study in Vancouver, Canada, where she took part in the Nutrition Research Program at the University of British Columbia. There she studied the uptake, incorporation and function of n-3 and n-6 fatty acids in primary neurons of rats.



After graduating in November 2005 she shortly worked at the company BioMerieux on a literature research on decontamination methods applicable in the NASBA manufacturing building. She also spent 4 months at the Cell Biology and Immunology department at the Wageningen University working on a project on summer eczema occurring in horses.

In September 2006 she started her PhD, the results and outcomes of which are described in this thesis. The research was carried out at the Cell Biology and Immunology Group and the Food Chemistry Group of the Wageningen University, under the supervision of prof. dr. Harry Wichers, prof. dr. ir. Huub Savelkoul and prof. dr. Clare Mills. The PhD project was partly funded by the EuroPrevall project, an EU-funded multidisciplinary project which aimed to improve quality of life for food allergic people. The objective was to analyse the impact of processing on the immuno-(allergo-) reactivity of allergenic proteins. As a second research topic, funded by FrieslandCampina and in cooperation with NIZO, several probiotic bacteria were screened and their potential immunomodulating capacity was investigated. Immunomodulation by probiotic bacteria could be beneficial in allergic diseases.

During her PhD project she was involved in teaching activities of three courses. Under her supervision seven students performed their theses in the subject of her PhD. Yvonne presented her work at several international conferences and organized a two-week 'PhD-study trip' to China. Furthermore she attended several international courses, thereby extending her knowledge and scientific network.

In September 2010 she was appointed to start up a project financed by Mead Johnson for the period of 6 months. This project investigates immunomodulatory dietary factors in dairy milk products for the treatment of cow's milk allergy. Currently, she has a temporary position as immunologist at the Life Science department of FrieslandCampina Research in Deventer.



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