

ORIGINAL ARTICLE

***Lactococcus lactis* harbouring Ara h 2.02 alleviates allergen-specific Th2-associated responses in sensitized mice**C.J. Chan¹ , Y.S. Yong¹ , A.A.L. Song², R. Abdul Rahim³, L.L.A. In¹ and R.L.H. Lim¹ ¹ Faculty of Applied Sciences, UCSI University, UCSI Heights, Cheras, Kuala Lumpur, Malaysia² Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, UPM Serdang, Selangor, Malaysia³ Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, UPM Serdang, Selangor, Malaysia**Keywords**

IgE alleviation, *Lactococcus lactis*, oral administration, peanut allergy, recombinant Ara h 2.02.

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Abstract

Aim: To study the prophylactic effect of recombinant *Lactococcus lactis* (rLl) harbouring Ara h 2.02 peanut allergen, in sensitized and challenged mice.

Methods and Results: Ara h 2.02 cDNA was cloned into pNZ8048 for heterologous expression in *L. lactis*. The purified recombinant allergen showed IgE binding comparable with native Ara h 2. Balb/c mice were fed with either recombinant (rLl), nonrecombinant *L. lactis* (Ll) or NaHCO₃ (Sham) prior to sensitization and challenged with rAra h 2.02, whereas the baseline group was only fed with Ll. Allergen-specific immunoglobulin and splenocyte cytokines responses were determined for each mouse. Mice fed with either Ll or rLl showed significant alleviation of IgE and IgG₁ compared to the Sham group. Despite no significant decrease in Th2 (IL-4, IL-13, IL-6) or increase in Th1 (IFN- γ) cytokines, both groups showed lower IL-10 level, while the IL-4 : IFN- γ ratio was significantly lower for rLl compared to Ll group.

Conclusions: Oral administration of rLl harbouring Ara h 2.02 demonstrated alleviation of Th2-associated responses in allergen-challenged mice and a possible added allergen-specific prophylactic effect.

Significance and Impact of the Study: Ara h 2.02 coupled with the intrinsic properties of probiotic *L. lactis* as a delivery vehicle can be explored for the development of a commercially scalable vaccine.

Introduction

Peanut allergy can be triggered by exposure to tiny amounts of peanut allergens and in sensitized subjects this will lead to an IgE-mediated type-I hypersensitivity reaction driven by T-helper type-2 (Th2) immune response to the peanut allergens (Al-Mushem *et al.* 2003; Wang and Sampson 2011; Du Toit *et al.* 2016). It is one of the most severe and life-threatening food allergies, with increasing global prevalence. A 2.0–5.0% prevalence of peanut allergy was recorded in school-age children in the United States and recent research data presented at the American College of Allergy, Asthma and Immunology (ACAAI) Annual Scientific Meeting

2017, suggested an astounding 21% increase in peanut allergy in children since 2010 (Bunyavanich *et al.* 2014). A similar increase in the prevalence of peanut allergy was reported in the United Kingdom (Venter *et al.* 2010) and an increase in the incidence of peanut allergy among children and adolescents (ranging from 0.2 to 1.0%) has also been observed in Asian countries (Chiang *et al.* 2007; Shek *et al.* 2010; Lee *et al.* 2013; Yadav and Naidu 2015). Peanut allergy, like other food allergies, is most prevalent among those with an atopic predisposition, whereby children with multiple food hypersensitivities and a family history of atopic dermatitis are at a higher risk for developing peanut allergy (Shek *et al.* 2010).

The standard of care in peanut allergy is strict avoidance of peanuts and self-administration of epinephrine upon accidental exposure. Furthermore, it decreases the quality of life of patients and their families and is also considered as an economic burden in Western countries with high prevalence of peanut allergy (Sheikh and Burks 2013). Therefore, as an alternative to avoidance, safe and effective treatments are needed. Recent research has increasingly focused on tolerance induction and/or desensitization against peanut allergens as a means of prevention and treatment. Although oral immunotherapy (OIT), involving exposing allergic subjects to increasing dosages of peanut powder or crude extract, has shown promising results, it may not be suitable for patients who are at risk of adverse effects or anaphylaxis when exposed to higher doses of crude peanut or peanut flour (Khoriaty and Umetsu 2013). Recombinant peanut allergen, which is more consistent and specific compared to crude or native peanut allergen, is an alternative for component resolved diagnosis (CRD) and immunotherapy (Lorenz *et al.* 2001; Williams *et al.* 2008).

Peanut allergy is caused by an unusual sensitivity of the body's defence system towards the peanut allergens. Ara h 2.02, one of the two isoforms of the Ara h 2 major peanut allergen, is more potent compared to Ara h 2.01 due to an additional IgE epitope that confers higher binding and a more potent cross-linker of IgE (Chatel *et al.* 2003). Lehmann *et al.* (2003) reported the expression of Ara h 2 using the pET-32a and pET-16b vectors in *Escherichia coli* which resulted in mostly insoluble recombinant allergens. The solubility was improved to 50% when expression was performed in the disulphide promoting strains of *E. coli* Origami (DE3) and AD494 (DE3). Recently, we reported the expression of a codon-optimized Ara h 2.02 in *E. coli* using pET-28a (+) expression system that resulted in insoluble recombinant 6xHis-Ara h 2.02 which was purified and refolded by stepwise dialysis (Lew and Lim 2016). The refolded recombinant allergen was able to elicit a substantial level of allergen-specific IgE in sensitized *Balb/c* mice. In addition, the allergen-specific IgE elicited in the serum of these mice could bind to native Ara h 2, inferring that the recombinant Ara h 2.02 produced is allergenic and can be a potential reagent for diagnostic and therapeutic study.

Glenting *et al.* (2007) reported the expression of a soluble and secreted Ara h 2.01 in *Lactococcus lactis* p170/pAMJ399 expression system. Despite lower expression level in comparison to *E. coli*, lactic acid bacteria is the preferred expression system as it is generally regarded as safe (GRAS) and lacks endotoxin (lipopolysaccharide, LPS) (Kunji *et al.* 2003; Mierau and Kleerebezem 2005; Borrero *et al.* 2011). Furthermore, *L. lactis* is a promising candidate for the development of novel lactococcal-based

live oral vaccines due to its noncommensal nature, GRAS food bacterium that does not survive in the digestive tracts of animal models and humans, and its scalability for commercialization as probiotic food product (Drouault *et al.* 1999; Bahey-El-Din *et al.* 2008). Efficient expression vectors and host strains of *L. lactis* has been extensively engineered for the heterologous expression of therapeutic proteins (DNA, antigens, cytokines and enzymes) and for the development of novel mucosal delivery vaccines in prophylactic and therapeutic animal models (Bermúdez-Humarán *et al.* 2011). The efficiency of these oral vaccines for immunotherapy can be inferred by a shift from Th2 to Th1 response, an increase in IFN- γ and IgG_{2a} levels as markers for Th1 response with a corresponding decrease in allergen-specific Th2 cytokines (IL-4, IL-13, IL-6) and immunoglobulin, such as IgE and IgG₁, levels (Marinaro *et al.* 1997).

In this study, we aimed to heterologously express Ara h 2.02 in *L. lactis* using the pNZ8048/NZ9000 vector-host system and subsequently to evaluate the prophylactic effect of orally administered recombinant *L. lactis* harbouring Ara h 2.02 in allergen-sensitized and -challenged *Balb/c* mice.

Materials and methods

Ara h 2.02 expression construct

The Ara h 2.02 gene was amplified by PCR to generate a construct in pNZ8048, for secretion of recombinant Ara h 2.02 (S-Ara2.02-H). The PCR primers used are as shown in Table 1. USP45 signal peptide (80bp, ATGGTGAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCT) was incorporated at the N-terminal of the Ara h 2.02 gene using a two-step PCR. Overlapping primers Pnz_Ara_sp_1f, Pnz_Ara_sp_2r and Pnz_Ara_sp_3f and the previously cloned Ara h 2.02/pGEMT template (Lew and Lim 2016) were used in a primary PCR (30 cycles, denaturation at 95°C for 1 min, annealing at 68°C for 1 min and elongation at 72°C for 1 min, for 30 cycles) followed by a secondary PCR using Pnz_Ara_sp_1f and PnzAra_spHis_r primers. A 6xHis tag preceding a STOP codon was incorporated in the reverse primer PnzAra_spHis_r and a *Nco*I and a *Hind*III site was incorporated into the forward and reverse primer, respectively, to facilitate cloning into the pNZ8048 vector as previously described (Holo and Nes 1989). Transformed *L. lactis* cells were plated on M17 agar containing 10 μ g ml⁻¹ chloramphenicol and incubated at 30°C for 2–3 days. Colonies were screened by PCR using the pNZ8048-specific primers (Table 1) and positive clones were verified by DNA sequencing.

Table 1 Primers for generation of Ara h 2.02 construct in pNZ8048

Primer	Sequence (5' to 3')
S-Ara2.02-H	
Pnz_Ara_sp_1f	CCCCCATGGTGAAAAAAGA TTATCTCAGCTATTTAATG
PnzAra_spHis_r	CCCCCAAGCTT TCAGTGATGATGATG ATGATGGTAGCGGTCTCGGCCGCC
USP45 signal peptide	
Pnz_Ara_sp_1f	CCCCCATGGTGAAAAAAGAT TATCTCAGCTATTTAATG
Pnz_Ara_sp_2r	TGACAACGGGGCTGCAGCAGAA AGTATCACTGTAGACATTAATAAGC
Pnz_Ara_sp_3f	GCCCCGTTGTACAGGTGTTACGC TGCCAAGCTCACCATACTAGTAGCC
DNA sequencing primers	
pNZ8048_f	TATTGTCGATAACGCGAGCA
pNZ8048_r	CGTTTCAAGCCTTGTTTTC

The *Nco* I and *Hind* III restriction sites are underlined. The 6xHis tag sequence in the reverse primers is indicated in bold, whereas the following STOP codon is indicated in bold and italicized.

Expression study of Ara h 2.02 in *L. lactis* and Western immunoblot analysis

Initial small-scale expression study of the S-Ara2.02-H/pNZ8048 in *L. lactis* was performed as previously described (Siaw *et al.* 2016). Briefly, 1 ml of overnight culture of the recombinant *L. lactis* in M17 broth was used to inoculate 50 ml M17 broth (10 µg ml⁻¹ chloramphenicol). Cells were allowed to grow at 30°C until OD_{600nm} reached 0.5, after which expression was induced with nisin (MoBiTec GmbH, Göttingen, Germany) at 40 ng ml⁻¹. Uninduced and induced culture samples of 1 ml each were obtained at hourly time points ($t = 0$ to 6 h). The culture samples were centrifuged at 12 100 *g* for 2 min and the cell pellet was boiled in 200 µl 1x SDS dissociation buffer (60 mmol l⁻¹ Tris-HCl (pH 6.8), 25% glycerol, 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol and 0.05% (v/v) bromophenol blue) at 95°C for 10 min, prior to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. To detect the presence of secreted recombinant protein, one volume of 20% trichloroacetic acid (TCA) was added to one volume of spent culture medium (at 4 h after induction of expression) and incubated on ice for 30 min. The mixture was centrifuged at 16 000 *g* for 15 min, the precipitated protein pellet was washed in 200 µl of cold acetone. To detect for intracellular expression, 50 ml of expression culture (at 6 h after 40 µg ml⁻¹ nisin induction) was sonicated as described by Tsumoto *et al.* (2003). Soluble supernatant was separated from the pellet and the pellet was lysed in 10 ml denaturing buffer (DB: 8 mol l⁻¹ urea in PBS) overnight at room temperature. The supernatant and solubilized pellet was analysed on SDS-PAGE and

Western immunoblot analysis was performed using the HisDetector™ kit (KPL, Gaithersburg, MD).

Purification, refolding and identification of rS-Ara2.02-H

Affinity purification of the 6xHis tagged recombinant rS-Ara2.02-H produced in *L. lactis* was performed using the Ni-NTA chromatography under denaturing condition as previously described (Lew and Lim 2016). Ni-NTA resin was equilibrated with 10 ml denaturing buffer (PBS with 8 mol l⁻¹ urea, 1% Triton-X 100, 10 mmol l⁻¹ imidazole and 20 mmol l⁻¹ β-mercaptoethanol) pH 7.8. Cells were sonicated and a solubilized pellet containing the recombinant protein was incubated with Ni-NTA resin for 1 h at room temperature. Flow-through from the column was collected and the resin was washed with 4 ml denaturing buffer at pH 7.8, followed by 5 ml denaturing buffer at pH 6.5, pH 6.3 and pH 5.3, respectively, and finally the purified recombinant protein was eluted in 4 ml elution buffer at pH 4.5 and analysed on SDS-PAGE. The purified rS-Ara2.02-H was dialysed and refolded by stepwise dialysis in 6, 4, 2, 1 mol l⁻¹ urea solution and PBS (30% glycerol) respectively. Refolded recombinant rS-Ara2.02-H was quantified using Bradford assay (BioRad, Hercules, CA) and stored at -20°C until use.

rS-Ara2.02-H identification and *in vitro* allergenicity study

The purified rS-Ara2.02-H recombinant protein was identified by LC-MS/MS (QTOF) analysis as previously described using an Agilent 1260 HPLC-Chip/MS Interface coupled with Agilent 6550 Accurate-Mass Q-TOF LC/MS. Generated raw data were searched against the corresponding genome database using the Agilent Spectrum Mill MS Proteomics Workbench software packages. The purified rS-Ara2.02-H, rAra h 2.02 (produced in *E. coli*) and native Ara h 2 (nAra h 2; ProteinLabs, San Diego, CA) were tested for their ability to bind serum IgE in pooled sera from mice sensitized with rAra h 2.02, as previously described (Lew and Lim 2016). Briefly, a 96-well microtitre plate was coated with 5 µg ml⁻¹ of rS-Ara2.02-H, rAra h 2.02 or nAra h 2 and enzyme-linked immunoassay (ELISA) was performed using the pooled sera from mice and secondary antibody of anti-mouse IgE-biotinylated (1 : 1000) followed by streptavidin alkaline phosphatase (1 : 2000).

Prophylactic regimen

Female *Balb/c* mice (6–8 weeks old) were acclimatised under controlled environment of regulated temperature, ventilation and ambient humidity. The protocol for animal experiment was approved by the Ethics Committee of

Faculty of Applied Sciences, UCSI University (Proj-FAS-EC-15-19). A modified regimen of the Ara h 2.02-induced sensitized mouse model which was developed previously was used to evaluate the prophylactic effect of recombinant *L. lactis* harbouring rS-Ara2.02-H (Lew and Lim 2016). The prophylactic regimen (Fig. 1) was tested on four mice groups (six per group) comprising of a baseline group of mice fed with NZ9000 *L. lactis* (LI), a positive control (Sham) group of mice fed with NaHCO₃ and test groups of mice fed with LI and recombinant *L. lactis* (rLI) respectively. The baseline group was sensitized and challenged with NaHCO₃ while mice from the other three groups were sensitized and challenged by subcutaneous injection with 7 µg per 100 µl per mouse of rAra h 2.02 (*E. coli* produced) (Lew and Lim 2016). A total of 200 ml of culture of *L. lactis* or recombinant *L. lactis* (LI or rLI) in M17 medium was grown to OD_{600nm} 0.5 and induced with 40 ng ml⁻¹ nisin for 6 h. The culture was pelleted by centrifugation at 1331 g for 10 min and the pellet was resuspended in NaHCO₃ to 10⁹ CFU per 100 µl, estimated based on OD_{600nm} of 0.4 equivalent to 10⁸ CFU per ml bacteria suspension (Timmer *et al.* 2006). Briefly, each mouse received one dose of 100 µl (10⁹ CFU) of LI or rLI or 100 µl NaHCO₃ per day, for three consecutive days in a week, for a total of six doses/mouse, orally administered using sterile gavage needles (Popper & Sons Inc., NY). Mice were then sensitized on days 30, 44 and 58 and challenged on day 92 by subcutaneous injections of rAra h 2.02. On the fourth day after being challenged, mice were sacrificed by cervical dislocation and spleen of each mouse was obtained for splenocyte cytokine profiling.

Detection of Ara h 2.02-specific immunoglobulin responses

The specific IgE, IgG₁ and IgG_{2a} levels in the sera of mice were measured by ELISA as previously described (Lew and

Lim 2016). Briefly, 96-well microtitre plates were coated overnight at 4°C with 5 µg ml⁻¹ recombinant Ara h 2.02 protein or with Igκ light chain, in coating buffer of 0.1 mol l⁻¹ NaHCO₃, pH8.3. Plates were washed once in PBST (0.05% Tween) and blocked with 3% BSA in PBST for 1 h and incubated with mouse sera (dilution of 1 : 5 for IgE; 1 : 200 for IgG₁; 1 : 100 for IgG_{2a}) at 4°C overnight. Standards of IgE, IgG₁ or IgG_{2a} (50–0.0244 ng ml⁻¹ by two-fold dilutions) (Pharmingen, San Diego, CA) were added to the wells which were coated with Igκ light chain, used to generate the respective standard curve. Plates were washed three times in PBST and incubated with biotinylated anti-mouse IgE, IgG₁ and IgG_{2a} (Pharmingen) (1 : 2000 dilution in 1% BSA) for 1 h at room temperature. After washing in PBST, streptavidin alkaline phosphatase (1 : 2000 dilution) was added and incubated for 1 h at room temperature and finally developed in 200 µl of SIGMAFAST™ p-Nitrophenyl phosphate (pNPP) substrate (Sigma, St. Louis, MO). Absorbance was measured at OD_{405nm} using the FLUOstar Omega ELISA plate reader (BMG Labtech, Offenburg, Germany). The specific IgE, IgG₁ and IgG_{2a} (ng ml⁻¹) levels were determined from the respective Ig standard curve.

Splenocyte cytokine profiling

Splenocyte culture was performed as previously described (Lim *et al.* 2009). Briefly, single-cell suspension was prepared from the spleen of each mouse, in supplemented RPMI 1640 (10% heat-inactivated fetal bovine calf serum, 2 mmol l⁻¹ sodium pyruvate, 100 U ml⁻¹ penicillin and 100 g ml⁻¹ streptomycin and 50 µmol l⁻¹ 2-mercaptoethanol; Gibco, Waltham, MA). Splenocyte (2 × 10⁵ cells per well) suspension from each mouse was respectively seeded in 96-well plate in duplicate, in the presence of 10 µg ml⁻¹ of rAra h 2.02 for 72 h and the culture supernatant was collected for cytokine profiling (TNF-α, IL-10, IL-4, IL-6, IL-13 and IFN-γ) using the Mouse Premixed

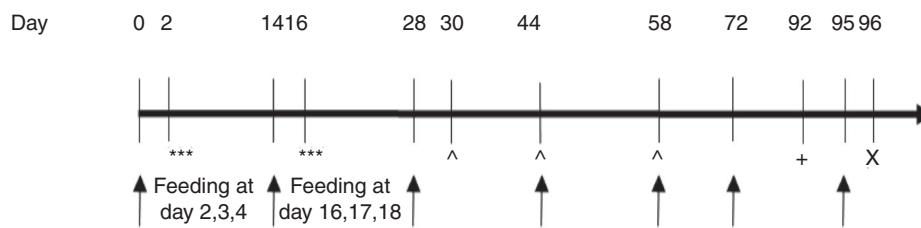


Figure 1 Prophylactic regimen. Balb/c mice were orally administered (*) with a total of six doses (1×10^9 CFU per dose), three doses in three consecutive days, for a duration of 2 weeks apart. Sham group ($n = 6$) mice were fed with NaHCO₃ and sensitized three times (^) and challenged (+) once by subcutaneous injection of 7 µg per mouse of rAra h 2.02 allergen. The baseline group ($n = 6$) mice were orally administered with *L. lactis* (LI) and subcutaneously injected with NaHCO₃ instead. Two test groups of mice fed with LI ($n = 6$) and recombinant *L. lactis* harbouring Ara h 2.02 (rLI, $n = 6$), respectively, were sensitized (^) and challenged (+) with rAra h 2.02 as described for Sham group. On the fourth day after challenge, mice were sacrificed and the spleen of each mouse was obtained for splenocyte culture and cytokine profiling (X). Blood samples were obtained through retro-orbital bleeding (↑) and used to determine the allergen-specific IgE, IgG₁ and IgG_{2a} responses.

Multi-Analyte Kit (R&D Systems, Minneapolis, MN) performed by Prima Nexus Sdn. Bhd., according to manufacturer's instruction.

Statistical analysis

All the statistical analyses and plotting of graphs were performed with the Microsoft® Excel (ver. 16.29.1). Data were expressed as mean $\pm$ standard deviation (SD) and statistical significance was tested using Student's paired *t*-test. $P < 0.05$ was considered as statistically significant.

Results

To determine the allergen-specific modulatory effect of recombinant *L. lactis* harbouring Ara h 2.02, an expression construct was generated by cloning the 615bp S-Ara2.02-H amplicon (Fig. 2a) into the *Nco* I and *Hind* III site of pNZ8048 expression vector. DNA sequence analysis verified the S-Ara2.02-H gene which was cloned in-frame with the vector's ATG initiation codon located in the *Nco* I site (CCATGG).

Small-scale expression study of S-Ara2.02-H/pNZ8048 in *L. lactis* at 3–24 h of induction with nisin, showed the presence of a 24 kDa rS-Ara2.02-H band in the cytoplasmic insoluble fraction instead of the soluble cell lysate or the TCA precipitated spent culture medium (Fig. 2b). Furthermore, the presence of this 24.8 kDa of rS-Ara2.02-H with its uncleaved USP45 signal peptide indicated the protein was produced in the cell and was not secreted. An attempt to facilitate refolding and secretion of rS-Ara2.02-H, by extending the duration of expression up to 24 h and decreasing the culture temperature from 30 to 22°C, was not successful as the recombinant protein remained intracellular and insoluble.

Scale-up expression culture (1 l) was performed using optimized condition of induction with 40 ng ml⁻¹ nisin at 30°C for 6 h. The rS-Ara2.02-H recombinant protein produced was purified under denaturing conditions using Ni-NTA affinity chromatography and eluted fractions containing the purified rS-Ara2.02-H protein (Fig. 2c) were pooled and refolded through stepwise dialysis, final dialysis was performed in PBS containing glycerol to enhance refolding (Yamaguchi and Miyazaki 2014). The yield of the purified and refolded rS-Ara2.02-H (Fig. 2c) was 159 mg l⁻¹ of expression culture. Tryptic digestion of this rS-Ara2.02-H using LC-MS/MS (QTOF) resulted in identification of nine peptides matched (coverage of 51.7% amino acid) with the amino acid sequence of annotated Ara i 2.02 (accession number A5Z1Q9) from *Arachis ipaensis* genome database (Table 2). The *A. ipaensis* (wild peanut) is highly identical to its direct descendant of the cultivated *A. hypogaea*.

The ability of a peanut allergen to elicit and bind allergen-specific IgE is indicative of its allergenicity. As shown in Fig. 3, rS-Ara2.02-H produced in *L. lactis*, rAra h 2.02 (previously produced in *E. coli*) and native nAra h 2 (Protein Labs) are comparable in their ability to bind specific IgE in serum of sensitized mice and did not show reactivity to control sera from nonsensitized mice. nAra h 2 contained a doublet protein band comprising of Ara h 2.01 and Ara h 2.02 isoforms (Hales *et al.* 2004).

After verifying the rS-Ara2.02-H produced by recombinant *L. lactis* (rLl), the prophylactic effect of rLl was evaluated on sensitized and challenged mice according to the regimen in Fig. 1. The Sham group initially showed low levels of Ara h 2.02-specific IgE and IgG₁ on days 0, 14, 28 and 44 but increased after the second subcutaneous sensitization with rAra h 2.02, on days 58, 72 and 95 (Fig. 4a,b). Compared to the Sham group, both groups of mice fed with rLl or Ll showed significant alleviation of allergen-specific IgE and IgG₁ during sensitization. No significant difference in the levels of IgE was observed between the Ll and rLl fed mice, except on day 72 whereby the rLl showed significantly lower IgE (Fig. 4a). In addition, the rLl fed mice showed significantly lower IgG₁ on days 72 and 95 compared to Ll-fed mice. Since IgG₁ is considered a Th2 immunoglobulin response, there is a possibility of a further attenuation of Th-2 responses conferred by mice fed with recombinant *L. lactis* (rLl). In accordance, on day 72, rLl-fed mice exhibit a significant decrease in IgG₁ : IgG_{2a} ratio compared to Ll-fed mice ($P = 0.040$) and Sham group ($P = 0.036$) and likewise on day 95 the ratio was lower for rLl-fed mice, while the Ll-fed mice ($P = 0.016$) and Sham ($P = 0.034$) group showed a significantly higher IgG₁ : IgG_{2a} ratio compared to the baseline group (Fig. 4d). A corresponding increase in specific IgG_{2a}, normally associated with a Th1 response, was observed in both the Ll or rLl compared to the Sham group. The baseline group as expected showed low levels of Ara h 2.02-specific IgE, IgG₁ and IgG_{2a}.

Splenocytes from these mice were cocultured with rAra h 2.02 and assayed for cytokine production to further support additional downregulation of Th2 responses in mice fed with rLl. Cytokine data shown in Fig. 5 showed that allergen-specific splenocytes of Ll ($P = 0.046$) or rLl ($P = 0.06$) group produced lower IL-10 compared to the Sham group. Despite this, rLl fed mice produced significantly higher TNF- α pro-inflammatory cytokine compared to Ll. The Th2-associated cytokines (IL-4, IL-13 and IL-6) level was lower for rLl compared to Ll and Sham group, although not significant. Furthermore, Th1 cytokine of IFN- γ was not significantly increased for both Ll and rLl compared to the Sham group. There was no observed difference in the IL-13 : IFN- γ ratio between

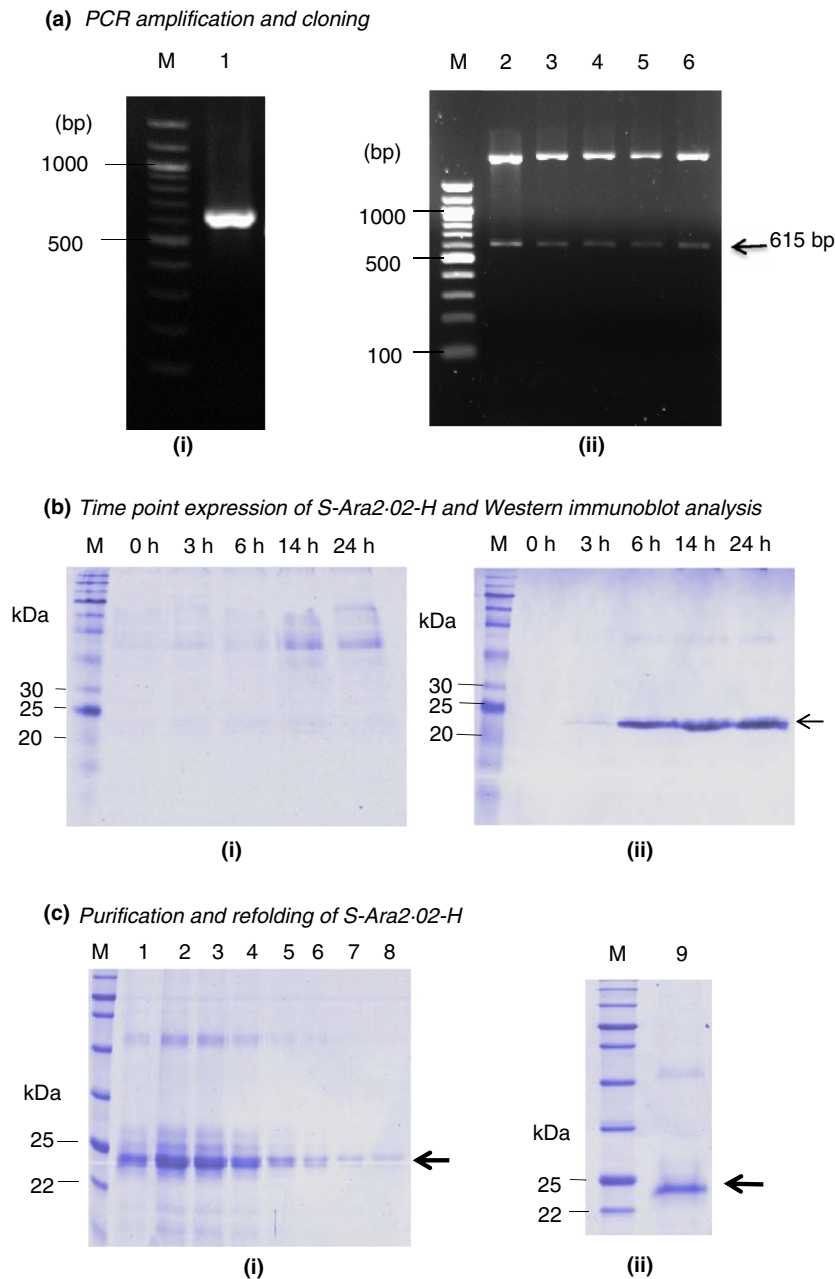


Figure 2 Generation of S-Ara2.02-H/pNZ8048 expression construct, expression and purification of S-Ara2.02-H in *Lactococcus lactis*. (a) The (i) PCR amplification and (ii) restriction enzyme digestion of S-Ara2.02-H/pNZ8048 plasmid clones. Lane M contained 100 bp DNA marker (NEB). Lane 1 contained 5 μ l PCR products of S-Ara2.02-H. Lane 2–6 contained *Nco* I and *Hind* III restriction enzyme digestions of positive clones of S-Ara2.02-H/pNZ8048 showing release of the 615 bp cloned S-Ara2.02-H gene (arrow). (b) Expression studies at different time points, before and after induction with 40 ng ml⁻¹ nisin, whereby (i) the SDS-PAGE analysis of 15 μ l of insoluble intracellular samples before induction ($t = 0$ h) and after induction at 3, 6, 14 and 24 h, respectively, did not show a distinct rS-Ara2.02-H band. However, (ii) the Western immunoblot assay for the insoluble intracellular samples at 3, 6, 14 and 24 h of expression showed a 24-8-kDa rS-Ara2.02-H protein band (arrow) whereby a faint recombinant protein band was observed at 3 h which increased in intensity at 6 h and above. No band was observed at 0 h. (c) (i) SDS-PAGE analysis containing 15 μ l of the respective fractions eluted at pH 4-5 from Ni-NTA affinity chromatography purification, indicating the purified 24-8 kDa rS-Ara2.02-H protein band (arrow) in lane 1–6, while (ii) lane 9 contained the purified and refolded rS-Ara2.02-H recombinant protein produced in *L. lactis*. Lane M contained prestained protein marker (P7706 and P7710 NEB).

Table 2 LC-MS/MS report of tryptic digested rS-Ara2.02-H. There were nine distinct matched peptides, with distinct summed MS/MS Search score of 163.33 and amino acid coverage of 51.7%

Accession No.	Annotated protein name	Species	Molecular mass (Da)/pI	Matched peptide fragments with annotated Ara h 2.02 (accession number A5Z1Q9) from Swiss Prot <i>Arachis</i> database
A5Z1Q9	Ara i 2.02	<i>Arachis ipaensis</i> (Wild peanut)	20 570.4/5.97	ANLRPCEQHLmQK RDPYSPSPYDR NLPQQCGLR DPYSPSPYDR DPYSPSQDPYSPSQDPDRR QQWELQGDR CmCEALQQImENQSDR CCNELNEFENNQR CmCEALQQIMENQSDR

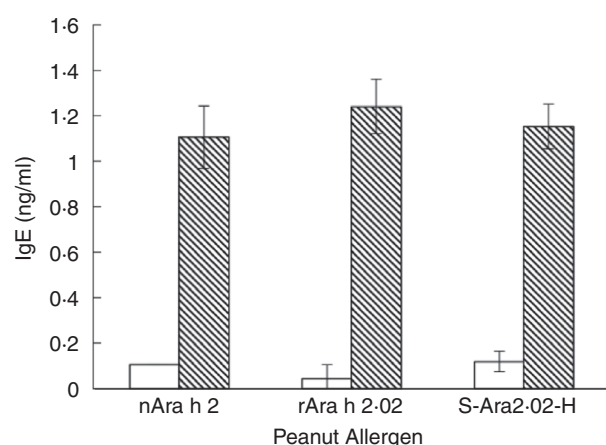


Figure 3 Recombinant S-Ara2.02-H, rAra h 2.02 and nAra h 2 native peanut allergen showed comparable reactivity to serum IgE. A total of $5 \mu\text{g ml}^{-1}$ of the respective allergens were used to coat a 96-well microtitre plate for ELISA assay, using pooled sera from peanut allergic mice (▨) and biotinylated anti-mouse IgE for detection. No significant difference was observed between the proteins in their reactivity to serum IgE, however, the reactivity to control sera from nonallergic mice (□) was shown to be at baseline level. Data obtained were from two technical and one biological replicate.

the three groups, but the IL-4 : IFN- γ ratio for rLI ($P = 0.0002$) was significantly lower than LI-fed mice (Fig. 5).

Discussion

The current approach on allergen immunotherapy (AIT) for type-I food allergy focuses on administration of allergens or extracts by oral, sublingual or subcutaneous route. However, frequent administration and the risk of adverse anaphylactic effect may limit its efficacy (Anagnostou *et al.* 2011; Varshney *et al.* 2011; Anagnostou *et al.* 2014). Specific strains of probiotic lactic acid

bacteria (LAB) has been shown to reduce Th2 allergic symptoms by inducing allergen-specific Th1 responses, this immunomodulation ability offers a promising concept for treatment of Th2-biased hyper-responsiveness (Bjorksten *et al.* 2001; Romagnani 2004). A number of studies have shown promising results combining a decade old probiotic concept with the use of LAB as delivery vehicle for different mucosal routes (Rautava *et al.* 2005; Bermúdez-Humarán *et al.* 2011). Adel-Patient *et al.* (2005) demonstrated that mice administered orally with recombinant *L. lactis* expressing bovine β -lactoglobulin were partially protected from sensitization. Based on this probiotic concept, we likewise evaluated the ability of recombinant *L. lactis* harbouring Ara h 2.02 as an oral delivery vehicle to induce Ara h 2.02-specific prophylactic effect in Ara h 2.02-sensitized and -challenged mice.

The S-Ara2.02-H/pNZ8048 construct was initially generated containing a USP45 signal peptide sequence derived from *L. lactis* for secretion of a recombinant Ara h 2.02 produced in *L. lactis*. Nisin, a food grade antibiotic, was used to induce expression of Ara h 2.02 gene in pNZ8048 vector, driven by the P_{nisA} promoter and facilitated by a nisin operon in the NZ9000 host's genome that also enabled the *lactis* NZ9000 cells to be resistance to nisin (Kuipers *et al.* 1993; Kunji *et al.* 2003). In our study, 40 ng ml^{-1} nisin was able to induce S-Ara2.02-H expression in *L. lactis* NZ9000, similar to the nisin concentration previously used for expression of an orchid fragrance in the same vector system (Song *et al.* 2012). However, our study showed that rS-Ara2.02-H was expressed intracellularly as insoluble recombinant proteins. The efficiency of USP45 signal peptide was reported to vary dependent on the nature of the expressed protein and culture conditions (Dieye *et al.* 2003; Hazebrouck *et al.* 2007; Siaw *et al.* 2016). Cano-Garrido *et al.* (2014) reported the solubility of aggregation-prone GFP variant (VP1GFP), expressed using pNZ8148 in *L. lactis*, was

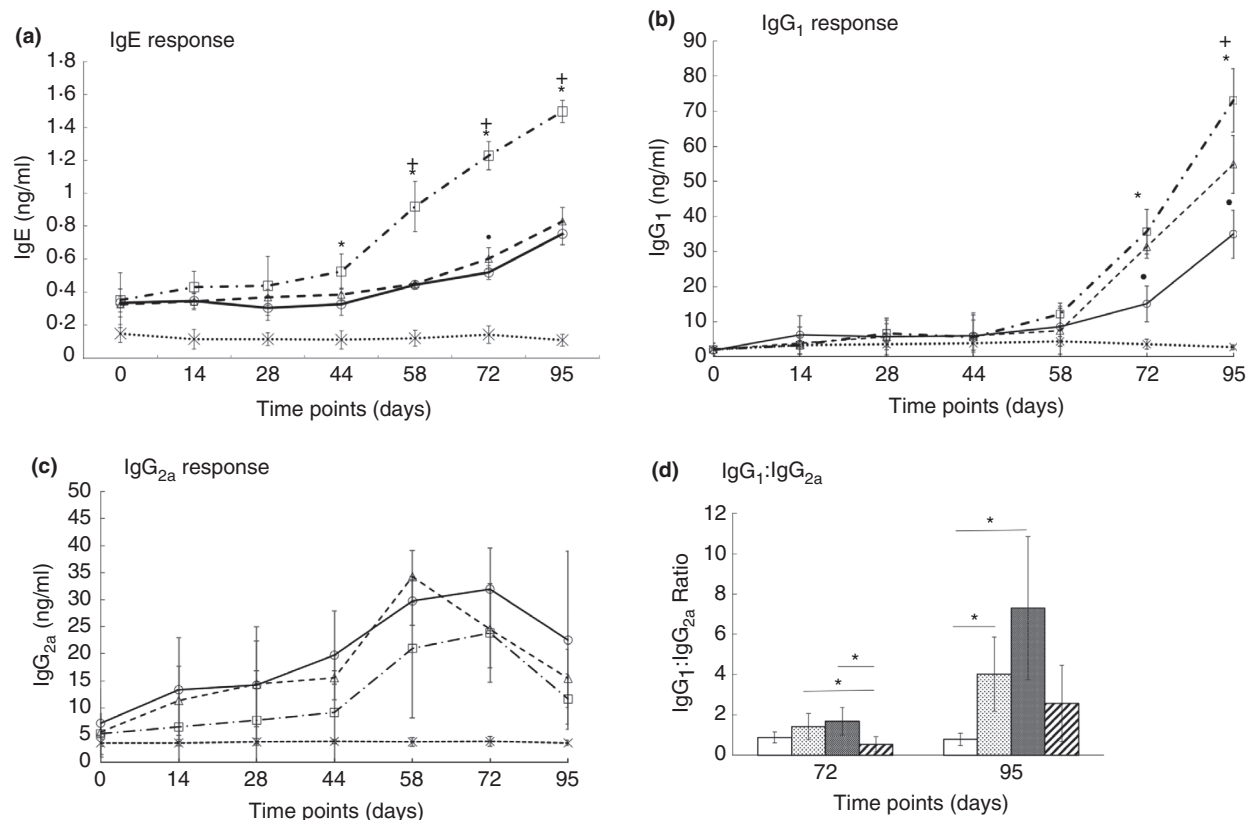


Figure 4 Ara h 2.02-specific IgE, IgG₁ and IgG_{2a} responses in mice. Blood samples of mice were collected on days 0, 14, 28, 44, 58, 72 and 95 and assayed for Ara h 2.02-specific IgE, IgG₁ and IgG_{2a} responses. The Sham group (NaHCO₃/rAra h 2.02) (---□) showed an increase in specific IgE and IgG₁ on day 58 after the first (day 30) and second (day 44) sensitization with rAra h 2.02 and the levels further increased after the third (day 58) and fourth (day 92) sensitization (4A and 4B). Both rLI (—○) or LI (—●) groups showed significant alleviation of allergen-specific IgE levels (on days 58, 72 and 95) compared to the Sham group, when sensitized with rAra h 2.02 (a). Significantly lower IgE (on day 72) and IgG₁ (on days 72 and 95) was observed in rLI compared LI group. A corresponding increase in IgG_{2a} production in mice fed with either LI or rLI was also observed (c). Baseline group (LI/NaHCO₃) (---×) showed baseline levels of specific Ig tested. For the IgE and IgG₁ response graphs, significant difference is indicated by (*) for Sham group compared with rLI-fed mice; (+) for Sham compared with LI-fed mice; and (●) for LI- compared to rLI-fed mice, $P < 0.05$. The IgG₁ : IgG_{2a} ratio for mice fed with rLI (▨) significantly decreased on day 72 when compared to LI (▤) ($P = 0.040$) and Sham group (■) ($P = 0.036$). On day 95, the IgG₁:IgG_{2a} ratio was significantly higher for LI ($P = 0.016$) and Sham group ($P = 0.034$) when compared to the baseline group (□) whereas there was no significant difference between rLI and baseline group after challenged with rAra h 2.02 (d). Data obtained were in duplicates and presented as mean $\pm$ SD.

influenced by time, culture conditions and growth temperature, which have a dramatic effect not only on protein yield but also on protein solubility and conformational quality that were particularly favoured under fermentative anaerobic metabolism. Despite testing an extended duration of expression (6–12 h) at different growth temperatures (including the optimum 30°C), as previously reported for secretion of recombinant proteins containing USP45 (Subramaniam *et al.* 2012; Siaw *et al.* 2016), the expressed recombinant S-Ara2.02-H remained insoluble and intracellular even after 24 h of expression. Subsequent purification and refolding of the insoluble rS-Ara2.02-H recombinant protein produced in *L. lactis* was performed to verify its identity and allergenicity prior to prophylactic study. This endotoxin free rS-Ara2.02-H produced in *L.*

lactis may be an alternative to native Ara h 2 for use in CRD since Ara h 2.02 allergen was reported to show high prevalence (81–90%) of binding to serum IgE in peanut allergic individuals (Hales *et al.* 2004).

To evaluate the prophylactic effect of recombinant *L. lactis* harbouring the allergenic rS-Ara2.02-H (rLI) on Ara h 2.02-sensitized and -challenged mice, appropriate positive and negative control groups are important. The baseline group of mice fed with *L. lactis* (LI) provided information on basal immune response, whereas the Sham group (positive control) evaluated the regimen for induction of Th2 allergy responses in the sensitized and challenged mice. Mice fed with LI prior to sensitization and challenge provided information on the prophylactic effect of recombinant *vs* nonrecombinant *L. lactis*. We

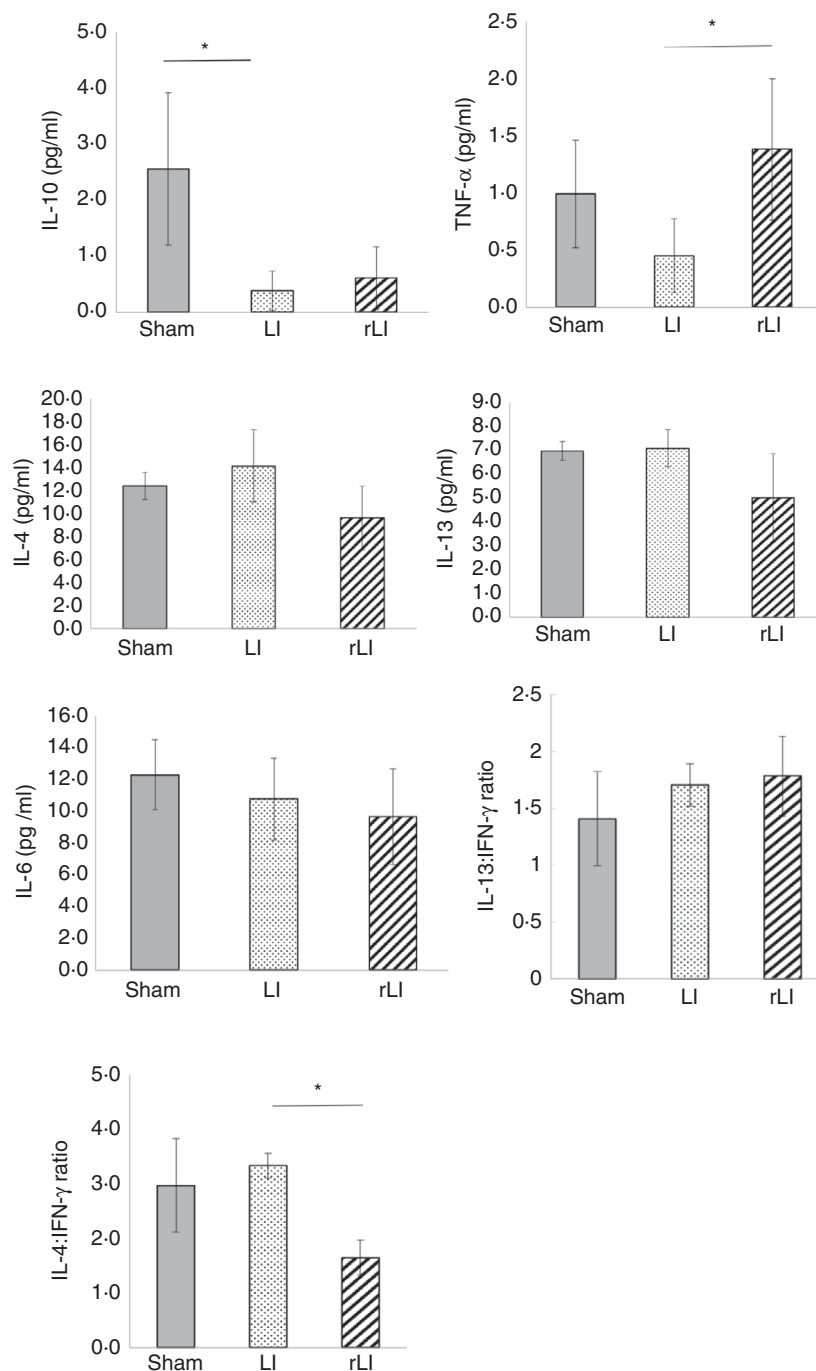


Figure 5 Splenocyte cytokine profile of experimental mouse. After the final challenge with rAra h 2.02, splenocytes from each mouse were cocultured with rAra h 2.02 for 72 h and culture supernatant was harvested for cytokine profiling. Cytokine data, adjusted for the baseline group, indicated that allergen-specific splenocytes from Sham group produced higher IL-10 cytokines compared that produced by LI-fed ($P = 0.046$) and rLI-fed ($P = 0.06$) mice splenocytes. On the contrary, pro-inflammatory cytokine TNF- α produced was significantly higher for rLI group ($P = 0.024$), albeit lower IL-10 level. No significant difference was observed in the IL-4, IL-13 and IL-6 cytokines levels between the groups. Although the IL-13 : IFN- γ ratio showed no difference between groups, the IL-4 : IFN- γ ratio for rLI-fed mice splenocytes was significantly lower compared to LI-fed mice ($P = 0.0002$). Significant difference ($P < 0.05$) between groups is indicated by (*).

previously reported the sensitization of Balb/c mice by subcutaneous injection of rAra h 2.02 either in the

presence or absence of alum adjuvant (Lew and Lim 2016). Since substantial levels of allergen-specific IgE and

IgG₁ responses were elicited in the absence of adjuvant; the adjuvant-free sensitization regimen was used in this study to rule out possible bias in immune response associated with the use of an adjuvant. Allergen-specific IgE is the mediator for most immediate clinical reactions of food allergy due to breach in oral tolerance (Chehade and Mayer 2005). It has been well established that the internal profile of Th1 and Th2 cytokines determines the orientation of antibody responses. Following allergen stimulation, Th2 cytokines, such as IL-4 and IL-13, can induce CD40-stimulated IgM⁺ IgD⁺ naïve B cells to undergo isotype switching to IgG₁ and IgE while retaining their allergen specificity and IL-13 can further enhance proliferation of these allergen-specific B cells (Coffman *et al.* 1991; Hasbold *et al.* 1999). In the context of AIT, a shift from Th2 to Th1 response is anticipated, whereby in the presence of Th1 cytokines such as IFN- γ , conversely regulates the Th2 responses by further suppressing Th2 cell activation and IL-4 secretion and promotes class-switch of CD40-stimulated B naïve cells to produce IgG_{2a} (Marinaro *et al.* 1997). IL-10 is a cytokine with pleiotropic effects on immunoregulation and inflammation. It is a cytokine derived from Th2 cells, plays a role in stimulating mast cells and enhances B-cell maturation and antibody production. IL-10 also suppresses pro-inflammatory cytokine (IFN- γ , IL-2, IL-3 and TNF- α) production by macrophages and Th1 cells and thus is a potent inhibitor of monocyte/macrophage antigen presentation capability. IL-6 plays important roles in autoimmunity and inflammation and is essential for Th2 cell differentiation.

As hypothesized, mice fed with *L. lactis* (either rLI or LI) showed significant attenuation of allergen-specific IgE levels upon challenged with rAra h 2.02, compared to Sham group. Furthermore, rLI showed significantly lower IgE (on day 72), IgG₁ (on days 72 and 95) and IgG₁:IgG_{2a} ratio compared to LI group, inferring a further decrease in Th2 immunoglobulin response conferred by rLI. Despite the significant shift from Th2 to Th1 immunoglobulin responses observed in LI- and rLI-fed mice, the downregulation of allergen-specific Th2 cytokines (IL-4, IL-13 and IL-6) and upregulation of Th1 (IFN- γ) cytokine were not significant. This may be due to the use of primary instead of secondary splenocyte culture for cytokine profiling, the latter tends to enrich allergen-specific T cells that may provide a more distinct cytokine profile. Nevertheless, IL-10 a Th2-associated cytokine was lower in mice fed with either rLI or LI compared to the Sham group. Furthermore, the splenocytes of rLI-fed mice produced lower levels of IL-4 and a significantly lower IL-4 : IFN- γ ratio compared to LI-fed mice.

Our result supported the previous study by Ren *et al.* (2014) who similarly observed a decrease in Th2-type IL-

4 levels in crude peanut extract (CPE)-stimulated splenocytes of mice fed with recombinant *L. lactis* expressing Ara h 2.02. The difference between our study and that of Ren *et al.* (2014) was the *L. lactis* expression vector (pNZ8148) and strains of mice (C3H/HeJ) used as well as the prophylactic regimen employed. The C3H/HeJ mice were fed daily with 2×10^9 CFU per mouse for a total of 14 days in the prevention phase compared to six times feeding (1×10^9 CFU per mouse) over a period of 2 weeks in our study. Furthermore, the sensitization and challenge protocol used CPE in combination with 10 μ g of cholera toxin (CT) compared to our protocol using rAra h 2.02 allergen which allowed for determination of the Ara h 2.02-specific immunoglobulin and cytokine responses. Albeit the differences, our data are corroborated with the modulation of allergic immune responses conferred by oral administration of recombinant *L. lactis* harbouring Ara h 2.02 (rLI) in subcutaneously sensitized and challenged mice.

Significantly higher production of pro-inflammatory cytokine TNF- α was observed in rLI compared to LI-fed mice and Sham group, albeit a significantly lower IL-10 level compared to Sham group. Miettinen *et al.* (1996) reported that live LAB (including *L. lactis*) but not glutaraldehyde-fixed LAB were potent inducers of pro-inflammatory TNF- α and IL-6 cytokines production by human peripheral blood mononuclear cells (PBMC), even higher than that induced by lipopolysaccharide (LPS). This implies the importance of live LAB for oral vaccine development due to its inherent adjuvant nature to stimulate immune responses. Furthermore, different strains of LAB varied in their immunomodulation ability, attributed by their different cell wall structures such as capsular polysaccharides, peptidoglycans and lipoteichoic (Sato *et al.* 1988). The size and tertiary configuration of peptidoglycan of Gram-positive LAB is important for induction of TNF- α release (Timmerman *et al.* 1993). In our study, the increase in TNF- α production by allergen-specific splenocytes of rLI-fed mice could result from the immune defence against rS-Ara2.02-H delivered by *L. lactis*. Contrary, IL-6 and IL-10 cytokine produced by these mice is lower than Sham group. IL-10, an important regulatory cytokine, with an anti-inflammatory and immunosuppressive role, has been reported to restrain allergic and asthmatic disease by balancing the Th1/Th2-type responses and relieve allergic inflammation (Asadullah *et al.* 2003; Wu *et al.* 2007). However, our observation is in accordance with suppression of IL-10 levels observed by Ren *et al.* (2014) for mice fed with recombinant and non-recombinant *L. lactis*. Ren *et al.* (2014) also reported an induction of Th3-type cells associated with oral tolerance and control of allergic responses via upregulation of TGF- β and Foxp3 gene expression in splenocytes and

intestine of mice fed with recombinant *L. lactis* (Josefowicz *et al.* 2012; Ren *et al.* 2014). Although in our study we did not assay for TGF- β and Foxp3 gene expression, the role of Th3-type cells in modulating allergic responses observed in rLI-fed mice cannot be ruled out.

Lactococcus lactis has been previously used for management of food allergy. Tang *et al.* (2015) reported that feeding freeze-dried probiotics daily in combination with peanut OIT decreased peanut-specific IgE. Different immunization regimens and doses are crucial for the evaluation of the efficacy of recombinant LAB either as oral or intranasal delivery vehicle for mucosal-based vaccines. The orally administered live *L. lactis* (LI or rLI) suspended in NaHCO₃ buffer may be sufficient in protecting the probiotics passing through the highly acidic environment of the stomach, enabling a substantial amount to reach the small intestine for induction of local responses by the gut-associated lymphoid tissue which resulted in the observed immunomodulation responses. Adel-Patient *et al.* (2005) demonstrated the protective effect of oral administration of six doses of recombinant *L. lactis* expressing bovine β -lactoglobulin (BLG), a major cow's milk allergen, in Balb/c mice. An enhanced efficacy of these LAB strains producing high amounts of BLG allowed the delivery of high doses up to 4–70 mg per mice for IgE inhibition. Faria and Weiner (2005) reported that early feedings of high doses of allergens tend to induce oral tolerance in mice. In our study, a total of six doses of recombinant *L. lactis* (1×10^9 CFU per 100 μ l per dose) were administered, corresponding to an estimated 0.23 mg per dose or a total of 1.36 mg of rS-Ara2.02-H/mouse. The low dosage administered may also account for the nondistinct-specific Th1 response observed, which could be improved by either increasing the dosage or frequency of feedings. Since *L. lactis* is a noncolonizing LAB in the gut, any immunomodulatory induction will need a prolonged period of administration to enable proper induction of relevant subsets of Treg cells to significantly attenuate Th2 responses.

It has been well established that mucosal administration is able to modulate humoral immune response through antigen transportation to systemic lymphoid organs, preferentially via the lymphatics (Adel-Patient *et al.* 2005). Further study to determine the mucosal-specific IgA responses, induction of Treg and Breg cells in the draining mesenteric lymph nodes, can be performed to elucidate the protective mechanisms of rLI, whether towards a desensitization or tolerance induction. Similar to Treg, the Breg cells play a role in the maintenance of allergen tolerance during AIT through induction of Treg and production of anti-inflammatory cytokines such as IL-10- and TGF- β -mediated suppression of effector Th2 allergic responses (Mauri and Bosma 2012; Akdis

and Akdis 2014; van de Veen 2017). Studies show that the frequency of these allergen-specific B cells increases during peanut immunotherapy in humans towards tolerance induction (Patil *et al.* 2015; Hoh *et al.* 2016).

In conclusion, the oral administration of rLI harbouring intracellular allergenic Ara h 2.02 demonstrated was able to alleviate allergen-specific Th2-associated responses in allergen-sensitized and -challenged mice, despite no distinct shift to Th1 response through elevation of IFN- γ was observed. Future study can focus on enhancing the modulatory effect via increasing the dosage administered and on elucidating the protective and therapeutic mechanisms conferred by rLI. The present work provides further evidence on the potential of mucosal LAB vaccines in AIT of peanut allergy. The role of recombinant *L. lactis* in promoting induction and responses of Breg and Treg cells during AIT can be a potential strategy for developing targeted immunotherapy for peanut allergy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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