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Evaluation of *Lactococcus lactis* carrying active For t 2 protein in immunotherapy for *Forcipomyia taiwana* allergy in mice

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ABSTRACT

Background: *Forcipomyia taiwana* (Diptera: Ceratopogonidae) allergy is Taiwan's most prevalent biting insect allergy. Our previous studies identified and cloned major *F. taiwana* allergens For t 2 and determined its strong immunogenicity to human fibroblasts. This study investigated whether oral administration of food-grade *Lactococcus lactis* containing For t 2 ameliorated midge-allergic symptoms in a mouse model.

Methods: BALB/c mice, divided into viable, sonicated, and autoclaved groups, were fed with *L. lactis*-For t 2 5 times weekly for 4 weeks. Immune molecules related to allergies in serum were analyzed and 16S rRNA profiles of fecal samples were compared.

Results: The results revealed administration of *L. lactis*-For t 2 significantly decreased the level of total IgE and ameliorated midge allergen-challenge-induced scratch bouts found in the viable and sonicated groups, but not the autoclaved group. The sonicated group showed a significant reduction in IL-4, IL-13, IL-17, MCP-1, and TNF-α levels. Its effect was comparable to that of the viable group and was even more pronounced in reducing IL-9 expression. Further, we performed a comprehensive analysis of 16S rRNA profiles corresponding to fecal samples. Clostridiaceae and Clostridium were the dominant family and genus in the autoclaved group, whereas Duncaniella and Kineothrix were the dominant genus in the sonicated and viable groups, respectively.

Conclusions: The results imply that mucosal allergen-specific immunotherapy of *L. lactis* For t 2 is a better cost-effective alternative to conventional subcutaneous allergen-specific immunotherapy. This study shows that non-viable *L. lactis*-derived For t 2 active protein may be a promising therapeutic for treating midge allergy.

Keywords: Ceratopogonidae, Lactococcus lactis, Sonication, Immunotherapy

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INTRODUCTION

Forcipomyia taiwana is a tiny hematophagous midge which is widely distributed throughout Taiwan.[1] Its larvae live in moderately moist soil around housing or shaded areas of cultivated bamboo, tea, and vegetables.[2,3] Moreover, the hematophagous midges of the same genus have also been reported worldwide, from tropical to temperate regions.[4–6] *F. taiwana* allergy is Taiwan's most prevalent biting insect allergy. As many as 60% of exposed individuals develop intense itchy reactions to the bites.[1,7,8] Among the 11 identified allergens, For t 2 is the most predominant allergen for *F. taiwana,* with 75% of midge-allergic patients showing specific IgE to For t 2.[7,8]

Our previous studies identified and cloned major *F. taiwana* allergens For t 2 and determined its strong immunogenicity to human fibroblasts.[7] We used For t 2 DNA as the de-sensitization vaccine to demonstrate that injectable or epicutaneous needleless vaccines can prevent and treat mice allergic to this annoying biting midge.[9–12] However, clinical trials of DNA-based vaccines will require considerable time and effort. Alternatively, we could develop a simple strategy to help midge-allergic subjects become much less sensitive to midge bites. This could potentially allow a more moderate approach to controlling the population of *F. taiwana*. We aimed to develop a food-grade product containing *Forcipomyia taiwan* allergen For t 2, using *Lactococcus lactis* as a vector, to induce hosts to establish immune tolerance against For t 2 allergen.

Para-probiotics are inactivated, or dead, ghost probiotics that consist of non-viable microbial cells with cellular components and metabolites.[13] It has been reported that administered orally in adequate amounts, para-probiotics confer a benefit on animals and humans.[14,15] In this study, to compare viable and non-viable *L. lactis*-For t 2 biotherapeutics with regard to their anti-allergic effect, live cells were inactivated using sonication on ice. The results suggest that non-viable *L. lactis*-For t 2 is a promising functional food with an anti-midge allergic effect.

MATERIALS AND METHODS

Constructions and protein induction of pNZ8149-For t 2 clone in food-grade *L. lactis* NZ3900 strain

The plasmid pNZ8149 and *L. lactis* NZ3900 food-grade strain purchased from MoBiTec (Goettingen, Germany) were used to express intracellularly recombinant protein in this study. To construct the recombinant plasmid expressing the fusion genes under the control of the regulatory promoter nisA, the encoding genes of For t 2 from *Forcipomyia taiwana* (described in GenBank accession no. EU678971) were synthesized by modifying its sequence based on the optimized codon usage in *L. lactis*. The synthetic 990-bp fragment of For t 2 was then cloned into the *Kpn* I/*Pst*I sites of the pNZ8149 vector in-frame. The constructed plasmid was transformed into the *L. lactis* strain NZ3900 and plated onto Elliker plates according to the manufacturer's instructions (MoBiTec). The lactose-positive colonies appeared yellow in color after 48 h of incubation at 30°C.

The selected positive *L. lactis-For t 2* recombinant clone was propagated in an M17 medium containing 0.5% lactose as the sole carbon source. Briefly, the clone was grown until an OD_{600} of 0.15 was reached and induced with 150 ng/ml of nisin (Sigma, Missouri, USA) for an additional 1–5 h at 30°C. The harvested cells were monitored for protein expression by SDS-PAGE and immunodetection using lab-made rabbit For t 2 polyclonal antibody. The expressed For t 2 protein was determined by immunoblotting using purified *E. coli*-derived For t 2 protein as standards.

SDS-PAGE and western blot

Cell lysates of *L. lactis* disrupted by sonication were separated on 4–12% polyacrylamide gels by Laemmli's method. After electrophoresis, gels were fixed and stained with 0.2% Coomassie brilliant blue R250. The gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) for immunoblotting and blocked with 2% BSA. The membranes were then probed with rabbit anti-rFor t 2 polyclonal antibody followed by a peroxidase-labeled goat anti-rabbit IgG antibody (10000-fold dilution, Millipore). After another extensive washing, membranes were submerged

in Immobilon Western Chemiluminescent HRP substrate (Millipore WBKLS0500) for 1–5 min and sealed in foil. Signals were documented with a Gbox Chemi XX9 imager (Syngene, UK).

Batch fermentation of recombinant *L. lactis* For t 2 and preparation of non-viable cell lysate for feeding administration

The recombinant L. *lactis* strains NZ3900 harboring pNZ8149-For t 2 were cultured in a bench-top fermenter (Firstek, Taiwan) equipped with a digital pH controller, temperature-control system, dissolved oxygen sensor, and a blender. An inoculum comprising 1% (v/v) of the seed culture was transferred to the 5-L fermenter. The pH was controlled at 6.8–7.2 by automatically adding 2 N NaOH solution with a pump and a 40-rpm slow agitation was maintained to keep the broth homogeneous. Recombinant protein inducer nisin was added to the broth with optimal dosage and time determined by pre-experiments. The harvested cell pellets were washed with sterile PBS and divided into 3 equal portions for animal experiments. The first condition of the cell pellets, named the viable group, was fresh storage as an aliquot of 5×10^{10} CFU/ml in an ultra-freezer (−80°C). The second condition of the cell pellets, named the sonicated group, was ultrasound treatment (Sonics Vibra-Cell VCX-600, Artisan, IL, USA). The L. *lactis*-For t 2 was inactivated by submerging a cell disruptor probe in a tube filled with 3 ml of cell suspension cooling on ice at a sonic power of 180 W for 40 min. The third condition of the cell pellets, named the autoclaved group, was autoclaving at 121°C for 15 min, which yielded heat-killed *L. lactis*-For t 2.

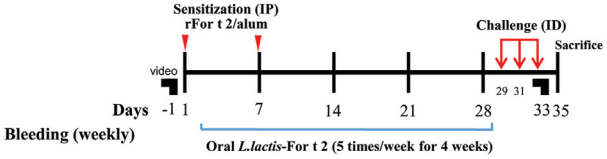
Preparation of *E. Coli*-expressed For t 2 recombinant protein for the murine model of sensitization

Recombinant For t 2 proteins were purified by rapid affinity column chromatography (Novagen, Darmstadt, Germany), followed by further removal of endotoxin with a polymyxin B column (Pierce, Rockford, IL, USA), and then sterilization with a 0.22-µm syringe filter (Millipore, Billerica, MA, USA), as described previously.[9,10,12]

Experimental design of *L. lactis*-derived midge biotherapeutics in a midge-allergic murine model

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital (La-1121982). Six-week-old female BALB/c mice from the National Laboratory Animal Center, Taiwan were used for the experiments. All mice were randomly assigned to 5 groups of 6 animals each.

Fig. 1 summarizes the experimental procedure. In brief, on days 1 and 7, mice were sensitized intraperitoneally (IP) with 10 µg/200 µl of rFor t 2 adsorbed to 1 mg alum adjuvant, according to a previously published model.[11,12] During days 2–28, mice from the 3 groups, ie, For t 2-viable, sonicated, and autoclaved, were intragastrically (IG) administered 200 µl of each of the respective *L. lactis*-supplements once a day on



	Group	N	Intra-peritoneal sensitization (Days 1 & 7)	Intragastric administration (200 µl) (Weeks 1-4 for 20 times)	Challenge (ID, 20 µl)
1	Naive	6	PBS	PBS	PBS
2	For t 2-Sham	6	10 µg rFor t 2 /1 mg alum	IG-PBS	1 µg rFor t 2
3	For t 2-viable	6	10 µg rFor t 2 /1 mg alum	IG viable NZ3900/pNZ8149-For t 2 (5×10^{11})	1 µg rFor t 2
4	For t 2-sonicated	6	10 µg rFor t 2 /1 mg alum	IG sonicated NZ3900/pNZ8149-For t 2 (5×10^{11})	1 µg rFor t 2
5	For t 2-autoclaved	6	10 µg rFor t 2 /1 mg alum	IG autoclaved NZ3900/pNZ8149-For t 2 (5×10^{11})	1 µg rFor t 2

Note: 1. Prior to oral, all mice were deprived of food for 2 h, but not water

Fig. 1 Schedule for L. *lactis*-derived midge biotherapeutics in a midge-allergic murine model. The BALB/c mice were intraperitoneally (IP) sensitized on day 1 and day 7 with recombinant For t 2 absorbed with alum. During days 2–28, mice were administered different processed *L. lactis*-For t 2 by gavage once daily on weekdays for 4 weeks. From days 29–33, all groups were intradermally (ID) challenged 3 times and sacrificed on day 35.

weekdays for a total of 20 times. On days 29, 31, and 33, all mice were challenged intradermally (ID) with rFor t 2. Skin-scratching behaviors were videotaped on day −1 to establish baseline data and on day 33 after the final ID challenge. Serum samples were collected from the submandibular vein bi-weekly and stored at −20°C until analysis. All mice were sacrificed on day 35, and skin, and spleen were removed for further study.

Measurement of serum total IgE and specific antibodies by ELISA

The serum levels of total IgE were quantified using a mouse IgE uncoated ELISA kit (Thermo Fisher Scientific, Vienna, Austria), according to the manufacturer's instructions. The levels of For t 2-specific IgE, IgG1, and IgG2a were determined by in-house ELISA. MaxiSorp plates (Nunc, Denmark) were coated with rFor t 2 for 2 h at 37°C. After washing with PBST, plates were blocked with 5% skimmed milk (for IgE) or 2% BSA (for all IgGs) for 2 h at room temperature. Sera were diluted (1:10 for IgE or 1:100 for all IgGs) in PBST and incubated at 4°C overnight (for IgE) or room temperature for 2 h (for all IgGs). For measurement of IgE, the plates were incubated with biotin-conjugated rat anti-mouse IgE (1:4000) for 2 h at room temperature. Subsequently, the plates were reacted with horseradish peroxidase-conjugated streptavidin (1:10,000) for 1 h, developed by adding TMB (Sigma), and stopped with 1 M H_3PO_4. For IgG measurement, the plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (1: 5000) or IgG2a (1:1000) for 2 h at room temperature and developed by adding ABTS solution (Sigma). The optical density was then analyzed on a Sunrise Absorbance Reader (TECAN, Austria) at 450 nm and 415 nm, respectively.

Evaluation of scratching behavior of immediate-type reaction in mice

On day 0 and day 33, the scratching behaviors were videotaped for 1 h starting immediately after the intradermal challenge with rFor t 2. Counts of scratching were made using video playback. Scratching behavior was observed as described previously.[11,12]

Histological examination of delayed-type reaction in skin lesions

On day 35, the mice were sacrificed and the abdominal skins from the challenge sites were removed and placed in 10% formalin overnight at room temperature. Briefly, tissues were embedded in paraffin, cut into 5-μm sections, deparaffinized, dehydrated, and stained with hematoxylin and eosin (H&E). Inflammatory cell infiltrates were examined by light microscopy and corresponding images were shot using an Olympus BX51 microscopic/DP71 Digital Camera System (Nagano, Japan).

Measurement of cytokine production in For t 2-stimulated splenocytes

Splenocytes were cultured in 24-well flat-bottomed plates at a concentration of 1×10^6 cells/ml and stimulated with 1 μg/ml of rFor t 2 or medium alone at 37°C for 3 days. The culture supernatants were collected and stored at −20°C until use. The levels of cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, Eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α were evaluated using a Bio-Plex mouse 23-plex panel (BioRad). Each protein concentration was determined based on a standard curve described by the manufacturer-provided protocol.

16S rRNA gene sequencing from mouse stool and data analysis

Microbial DNA was extracted from stool collected from all mice during days 33–35 using a QIAamp PowerFecal DNA kit (Qiagen, CA). 16S rRNA gene sequencing and data analysis were performed at the Research Technology Support Facility of BIOTOOLS (New Taipei City, Taiwan). In brief, 2 ng of gDNA was used for the PCR reaction with barcoded 16S gene-specific primers to amplify the full-length 16S genes (V1–V9 regions). The PCR products around 1500 bp were chosen and purified for SMRTbell library construction according to the Multiplexed SMRTbell procedure (Pacbio). Finally, sequencing was performed in the circular consensus sequence (CCS) mode on a Pacbio Sequel IIe instrument.

After demultiplexing, the CCS was further processed with DADA2 (version 1.10.1) to obtain amplicons with single-nucleotide resolution.[16] To analyze the sequence similarities among different amplicon sequence variants, we performed multiple sequence alignments using the QIIME2 alignment MAFFT against the NCBI database.[17–19]

RESULTS

Identification of recombinant For t 2 protein in viable and non-viable cell lysate of *L. lactis* NZ3900/pNZ8149-For t 2 clone

In this study, plasmid NZ8149 was engineered to express intracellular For t 2 protein in *L. lactis* strain NZ3900. The protein production was evaluated by Western blot using a lab-made anti-For t 2 polyclonal antibody. After a pilot study to determine the optimal dosage of nisin for induction, a protein band of approximately 36 kDa was observed gradually increasing from 1 to 3 h, but decreasing from the fourth hour (Fig. 2A). However, there was a non-specific band that appeared at the site of 40 kDa due to the polyclonal antibody reacting to the cell lysate of *L. lactis*. The yield of expressed rFor t 2 in engineered *L. lactis* was measured by immunoblotting using *E. coli*-derived rFor t 2 as the standards (Fig. 2A). The optimal level of *L. lactis*-expressed For t 2 was 100 ng/ml at induction with 150 ng/ml of nisin for 3 h. To obtain inactivated microbial cells, *L. lactis*-For t 2 was thermally inactivated by autoclaving for 15 min or sonication on ice for 40 min, The immunoblotting results are shown in Fig. 2B. The rFor t 2 protein was undetectable in heat-killed cell lysate. However, For t 2 was stable in sonicated cell lysate, which was kept at a low temperature, as detected in the lysate of viable cells.

The humoral immune effects of 3 *L. lactis*-For t 2 biotherapeutics on serum antibodies in sensitized mice

The effects of recombinant *L. lactis* on midge allergy, serum total IgE, and For t 2-specific IgE, IgG1, and IgG 2a levels were determined as previously described.[11] Fig. 3A shows that the total IgE concentration of the sham group on week 5 was elevated significantly compared with the naïve groups. Administration of engineered *L. lactis*-For t 2 significantly decreased the level of total IgE found in the viable and sonicated groups, but not the autoclaved group. The For t 2-specific IgE antibodies were reduced considerably only in the viable group (Fig. 3B) and the specific-IgG1 and -IgG2a levels were not significantly different among groups (Fig. 3C and D).

The effects of 3 *L. lactis*-For t 2 biotherapeutics on scratching behavior and histopathology of skin lesions in sensitized mice

The primary clinical requirement for midge-allergic patients is to provide relief from the annoying itchy allergic reaction to the midge bite. Administration of engineered *L. lactis*-For t 2 significantly ameliorated midge allergen-challenge-induced scratch bouts in both viable or sonicated groups compared with the sham group, but this effect was not observed in the autoclaved group (Fig. 4).

Fig. 5A depicts the histopathologic changes in the skin lesions in response to the midge allergen challenge. In the sham group, For t 2 allergen challenge significantly increased neutrophil, lymphocyte, and eosinophil cell count compared to the naïve mice (Fig. 5B). We found

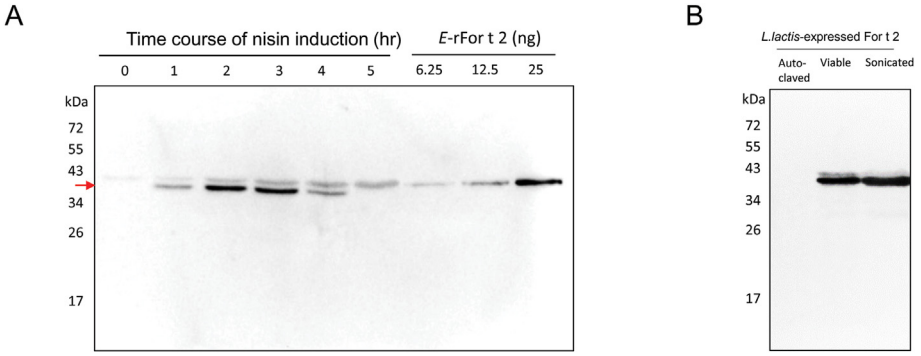


Fig. 2 Immunoblot analyses of *L. lactis*-expressed recombinant For t 2. (A) Intracellular expression of For t 2 protein was detected in total cell extracts after 150 ng/ml nisin induction for 1–5 h using For t 2-specific polyclonal antibody. *E. coli*-expressed For t 2 is used as the standard for quantification. (B) The L. *lactis*-expressed For t 2 was inactivated by autoclaving or sonication.

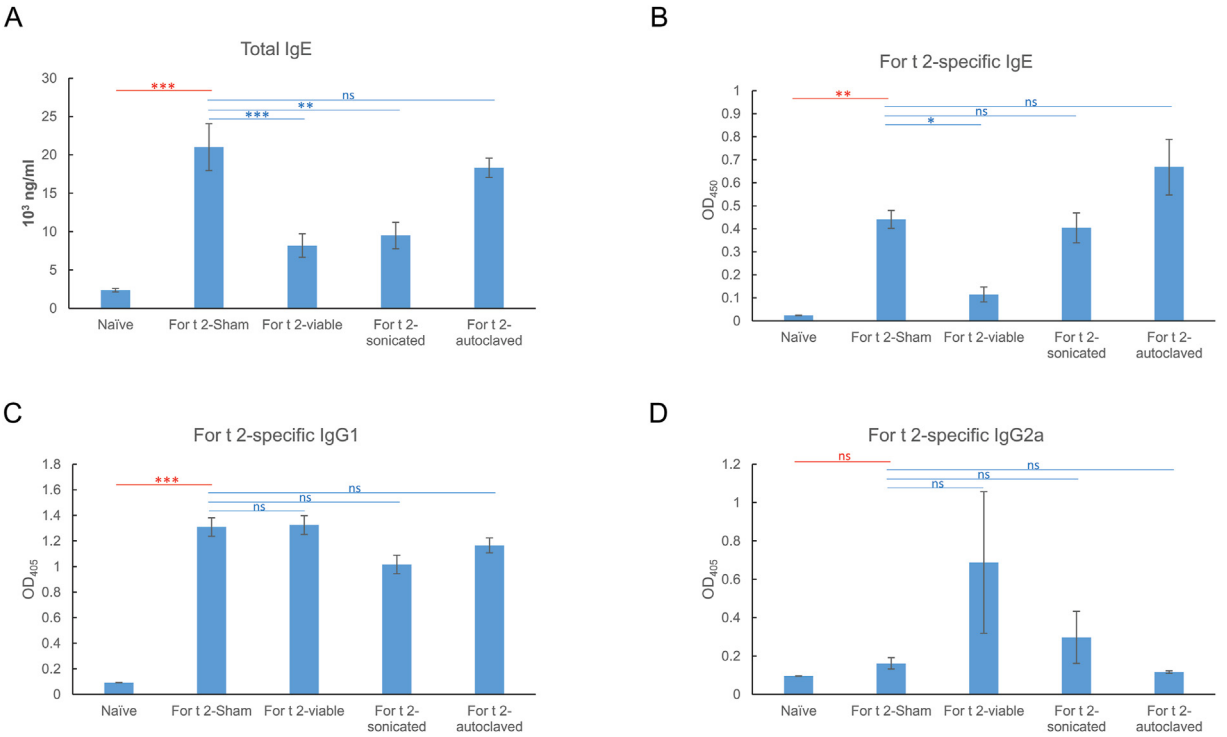


Fig. 3 Serum levels of total IgE (A), For t 2-specific-IgE, -IgG1 and -IgG2a (B, C, D) antibodies at week 5 determined by ELISA. Results are mean ± SEM of 6 mice from each group. The statistical significance of differences between groups was assessed by one-way ANOVA with Bonferroni multiple range test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns denotes no statistical significance.

that repeated oral feeding of viable or sonicated *L. lactis*-For t 2 significantly decreased eosinophil, neutrophil, and total cell infiltration in comparison with the sensitized mice. However, autoclaved *L. lactis*-For t 2 showed no efficacy, with no difference in cell recruitment compared with the sham group (Fig. 5A and B).

The effects of 3 *L. lactis*-For t 2 biotherapeutics on allergen-specific systemic inflammation using For t 2-stimulated splenocytes

Cytokine protein profiles from For t 2-stimulated splenocytes were analyzed with a multiplex immunoassay. As shown in Fig. 6, the sham group

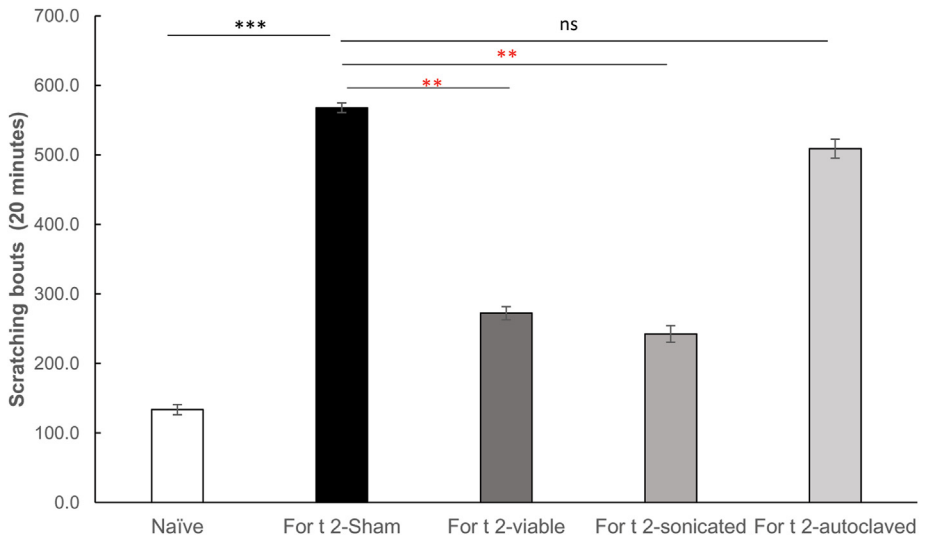


Fig. 4 Scratching bouts of mice from indicated groups. The scratching counts were recorded for 20 min after induction by intradermal injection of rFor t 2 at the endpoint of the experiment. Results are mean ± SEM of 6 mice from each group. **$p < 0.01$, ***$p < 0.001$, ns denotes no statistical significance by one-way analysis of variance with the Bonferroni multiple range test.

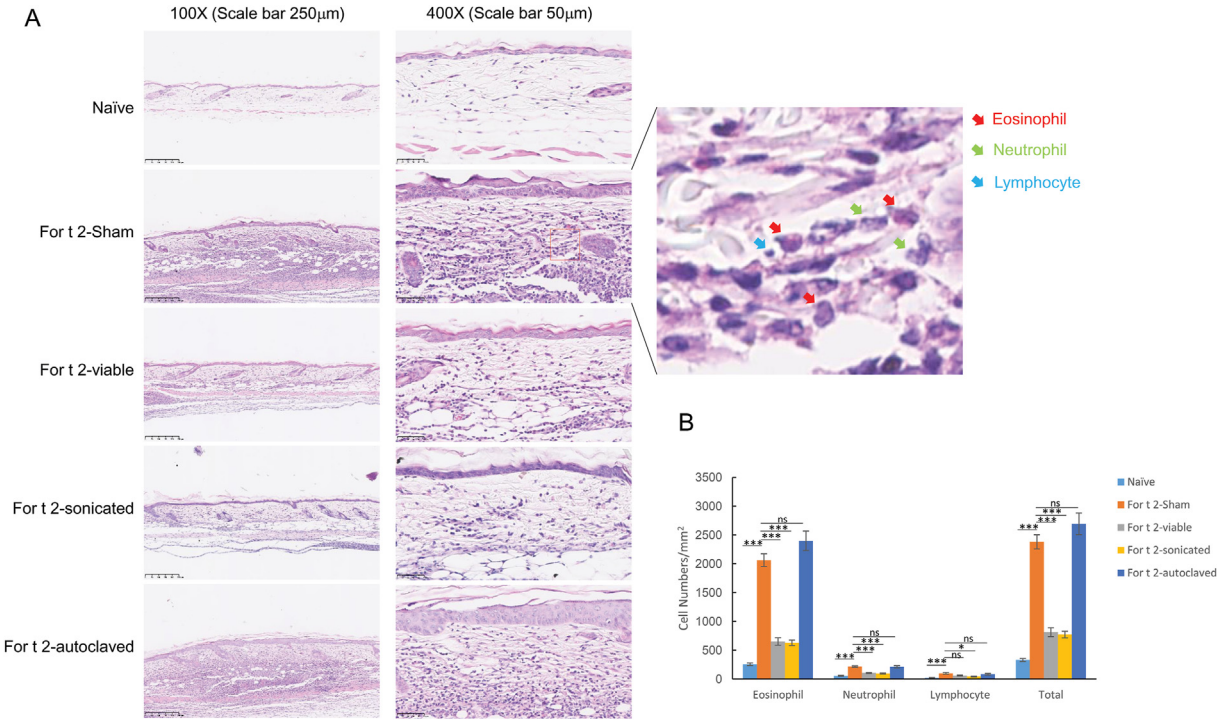


Fig. 5 Effects of feeding viable or non-viable *L. lactis*-For t 2 on histopathology of abdominal skins by H&E staining. Figures show the representative skin sections obtained 48 h after the intradermal challenge under a 100 × and 400 × light microscope. B. The infiltrating inflammatory cells were quantified under a 400-fold view from each mouse (n = 6 per group). The statistical significance of differences between groups was assessed by the Bonferroni multiple range test. *** denotes $p < 0.001$, ns denotes no statistical significance.

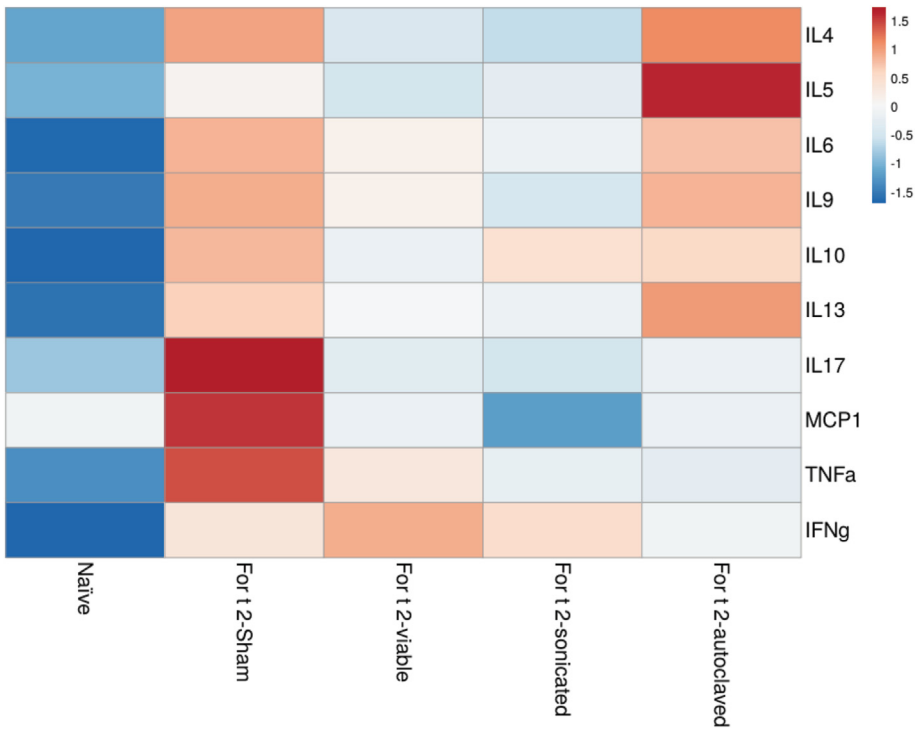


Fig. 6 Cytokine protein profiles of splenocytes in mice from each group. Mouse splenocytes were cultured under stimulation with For t 2 for 3 days. Culture supernatants were analyzed for cytokine release with Multiplex immunoassay. A heat map shows 10 statistically significant cytokines selected from the 5 groups. Color codes of the heatmap in each panel refer to fold changes with Z-score scales as the right-hand indication.

had significantly elevated protein levels of sera IL4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, MCP-1, TNF-α, and IFN-γ compared with the naïve group. Among them, mice that had been fed sonicated *L. lactis*-For t 2 showed a significant reduction of IL-4, IL-13, IL-17, MCP-1, and TNF-α levels compared to the sham group, with values comparable to those achieved in the viable group, and an even greater decrease in IL-9 expression. However, mice in the autoclaved *L. lactis*-For t 2 group showed a marked reduction of IL-17, MCP-1, and TNF-α compared to the sham group.

Comparison of the gut microbiota of mice after feeding 3 biotherapeutics of *L. lactis*-For t 2 for 4 weeks

To investigate how the different types of *L. lactis-For t 2* affected the gut bacterial community of mice, we sequenced 16S rRNA amplicons from the mice's feces after the 4-week oral gavage. In total, 15 fecal samples were included in our analysis (5 from each group with an average of 17401 non-chimeric reads per sample (±1578 SD).

We performed CPCoA analysis based on the UniFrac distance for all sample pairs to explore differences in gut microbiota composition among groups. The study revealed that CPCoA1 accounted for 57.21% and CPCoA2 accounted for 42.79% of the total gut microbiota variation among the 3 *L. lactis*-For t 2-fed groups. The analysis indicated that gut microbiota compositions from each group differed considerably (23.5% of variance, p = 0.001, Fig. 7A). Second, alpha-diversity metrics were performed to check whether the oral bacterial condition impacted their intestinal microbial diversity at the overall community level. Results from the analysis (Fig. 7B) indicated statistically significant differences in diversity metrics between the sonicated and viable groups (p = 0.0079). However, the differences in diversity metrics between the autoclaved and viable groups did not exhibit a statistically significant difference.

Subsequently, linear discriminant analysis effect size (LEfSe) was performed to identify the representative taxa in each group. Fig. 8 shows the predominant bacteria in the 3 groups at different levels under LEfSe. Clostridiaceae and Clostridium were the dominant family and genus in the autoclaved group, but were scant in the sonicated and viable groups. At the genus level, Duncaniella and Kineothrix were the dominant family in the sonicated and viable groups, respectively.

DISCUSSION

This study investigated the therapeutic potential of *L. lactis*-expressed For t 2 (a midge allergen) in different forms - viable, sonicated, and autoclaved – for treating midge allergy in a mouse model. The results revealed several important findings and implications for allergen-specific immunotherapy. Specifically, both viable and sonicated *L. lactis*-For

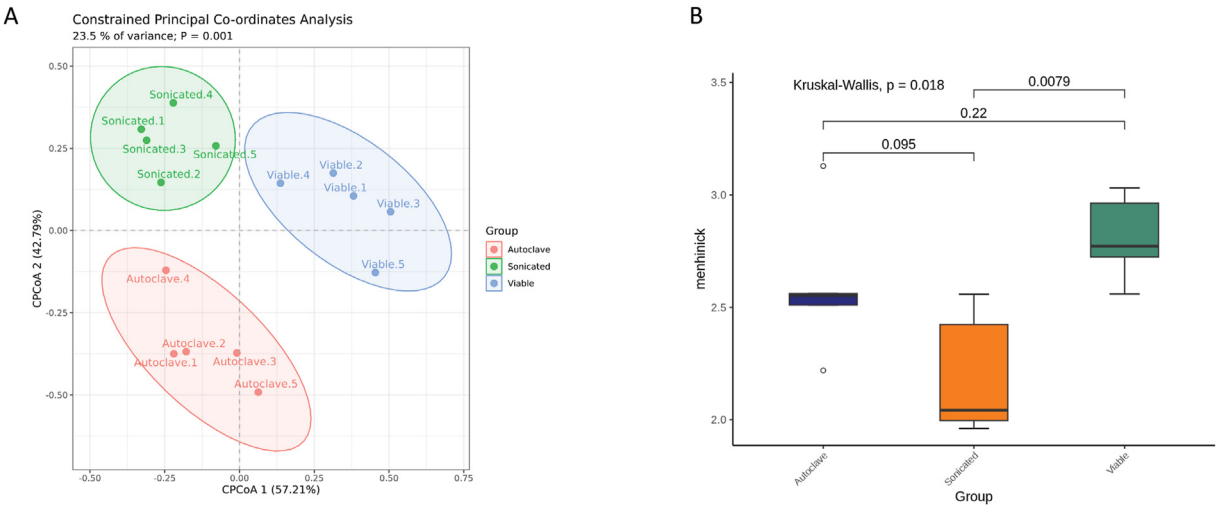


Fig. 7 (A) Constrained principal coordinate analysis (CPCoA) and (B) Alpha-diversity trends of gut microbiota from fecal samples of mice after feeding *L. lactis*-For t 2 for 4 weeks (n = 5 per group).

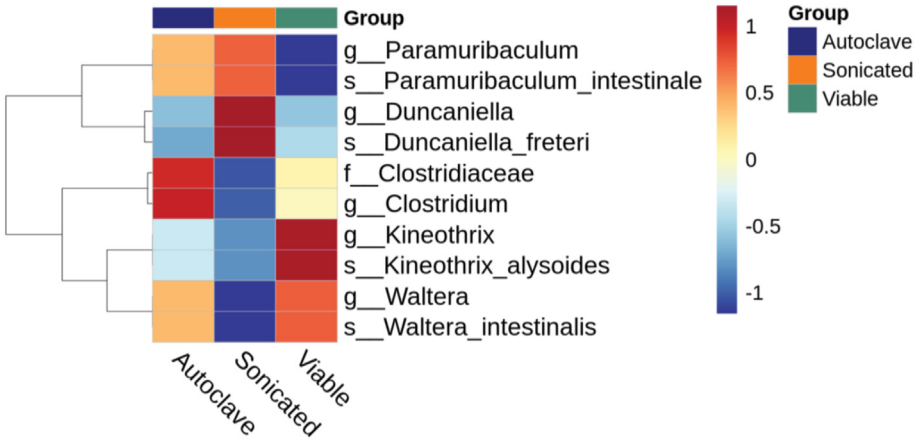


Fig. 8 Known taxa abundance reported by linear discriminant analysis effect size (LEfSe) in the bacterial community. Taxa with an LDA score >4 and $p < 0.05$ in the non-parametric factorial Kruskal-Wallis (KW) sum-rank test are shown. The heatmap presents a comparison of 3 *L. lactis*-For t 2-fed groups with relative abundance.

t 2 formulations significantly reduced total IgE levels and alleviated allergen-induced scratching behavior, while the autoclaved group showed limited efficacy. The sonicated formulation, which preserved the active For t 2 protein, was particularly effective in reducing pro-inflammatory cytokines, such as IL-4, IL-13, IL-17, MCP-1, and TNF-α, indicating its potential as a non-viable therapeutic option for midge allergy.

Insect bite allergies are often mediated by excessive Th2 responses, leading to IgE production and allergic reactions.[20] Current vaccine development for insect bite allergies focuses on neutralizing insect saliva proteins to prevent pathogen transmission or allergic reactions.[21,22] In our study, we observed a reduction in total IgE levels in animals treated with either viable or sonicated groups. This finding is consistent with prior research showing that both probiotics and paraprobiotics can modulate immune responses and attenuate allergic sensitization.[13,14] The significant decrease in Th2-associated cytokines (IL-4, IL-13), and the Th17 cytokine IL-17, in the viable group further supports the notion that live *L. lactis* can induce immune tolerance to specific allergens.[23] Although vaccination skewed the Th1 Immunity, the level of IgG2a, which is associated with a Th1 immune response, in the sonicated group did not increase significantly. Possibly, the amount of antigen was too small to cause a significant increase in IgG2a. Further study is required.

Histopathological analysis of skin lesions provided additional evidence of the therapeutic effects of *L. lactis*-For t 2. The reduction in inflammatory cell recruitment likely contributed to the observed decrease in scratching behavior, as eosinophils and neutrophils are known to release pro-inflammatory mediators that exacerbate itch and tissue damage.[8,12] In contrast, the autoclaved group showed no improvement compared to the sham group, further highlighting the importance of preserving the structural and functional integrity of For t 2 (the major allergen) during the preparation of biotherapeutics.

The impact of *L. lactis*-For t 2 on gut microbiota composition represents a novel aspect of this study. The sonicated and viable groups exhibited distinct microbiota profiles, compared to the autoclaved group, with an increased abundance of *Duncaniella* and *Kineothrix* associated with anti-inflammatory and immunomodulatory effects.[16] In contrast, the autoclaved group showed a predominance of *Clostridiaceae* and *Clostridium*, which are often linked to pro-inflammatory conditions. These findings suggest that the immunomodulatory effects of *L. lactis*-For t 2 may influence systemic immune responses through the gut-skin axis.

This study also highlights the translational potential of *L. lactis*-For t 2 as a cost-effective alternative to conventional subcutaneous allergen-specific immunotherapy. The oral administration of *L. lactis*-For t 2 represents a non-invasive and

patient-friendly approach to managing midge allergy, with implications for other allergen-specific immunotherapy applications. The scalability and safety of *L. lactis* as a food-grade vector further enhance its appeal as a platform for developing functional foods and biotherapeutics. Furthermore, the comparable therapeutic efficacy observed between sonicated and viable bacterial strains represents a notable finding with promising commercial implications. This equivalence in efficacy could potentially optimize the cost-effectiveness of large-scale production and distribution, as sonicated preparations circumvent the complex logistics and elevated costs typically associated with maintaining bacterial viability during storage and transportation.

Several limitations of this study warrant discussion. First, while providing valuable insights, the murine model of For t 2 allergy may not fully replicate the complexity of human allergic responses. Second, the long-term effects of *L. lactis*-For t 2 on immune tolerance and microbiota stability remain to be elucidated. Third, secretory IgA (sIgA) may offer valuable insights into gastrointestinal immune responses, especially given the oral administration route of allergen immunotherapy. To achieve a more comprehensive understanding of mucosal immunity in our model, we plan to incorporate sIgA analysis in future studies. Future studies should address these gaps by evaluating the durability of therapeutic effects and exploring the underlying mechanisms in greater detail. Additionally, clinical trials are necessary to validate the safety and efficacy of *L. lactis*-For t 2 in humans, paving the way for its potential commercialization.

In conclusion, this study demonstrates the therapeutic potential of *L. lactis* carrying For t 2 protein for managing *Forcipomyia taiwana* allergy. The findings highlight the efficacy of sonicated *L. lactis*-For t 2 in modulating allergic responses, reducing inflammation, and altering gut microbiota composition. These results provide a foundation for developing novel para-probiotic-based allergen-specific immunotherapies, offering a promising alternative to conventional approaches. Future research should focus on optimizing the formulation, understanding the mechanisms of action, and advancing the clinical translation of this innovative biotherapeutic product.

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Authors' contributions
MFL and YHC designed research; MFL, CHC, and CSW performed experiments; MFL, and NMW analyzed data; MFL, YHC, and NMW wrote the manuscript.

Declaration of competing interest
The authors have no conflict of interest to declare.

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