

Effects of Allergen-Specific and Non-Specific AGEs on the Allergenicity of Ovalbumin in a Mouse Model of Food Allergy

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Scope: Epidemiologic studies suggest a link between the incidence of food allergy and the consumption of dietary advanced glycation end-products (AGEs). However, the pathogenic role of dietary AGEs in food allergy is largely unknown. This study aims to investigate the effect of allergen-specific and non-specific AGEs on the allergenic manifestation of ovalbumin (OVA), a typical food allergen *in vivo*.

Methods and results: OVA is glycosylated by methylglyoxal to prepare allergen-specific AGEs (i.e., OVA-AGE), and a standard AIN-93G diet is heated to obtain allergen-non-specific AGEs. A BALB/c mouse model orally sensitized to OVA with different forms of AGEs is established and the outcomes are measured as clinical signs, specific antibodies, type-2/type-2 cytokines, immune cell subpopulations, intestinal barrier function, and gut microbiota (GM) composition. The OVA-AGE which has a lower immunoglobulin E (IgE)-binding level *in vitro* does not reduce the allergenicity of OVA but promotes a stronger T helper 2 cells (Th2)-response than native OVA *in vivo*. Both forms of AGEs up-regulate the expression of splenic RAGE and aggravate the destruction of gut barrier and GM dysbiosis, especially when exposed to non-relevant AGEs.

Conclusion: This study highlights the role of dietary AGEs in food allergy and helps to understand the biological consequences of immune-toxic compounds in modern diet.

Exploration of the important factors that drive food allergy has become a hotspot in recent years; however, the current explanations cannot firmly clarify the mechanisms of the etiology of the disease.^[1–3] Given that genetic variations are not enough to explain the rapid increase of allergic conditions in many areas, environmental factors such as dietary pattern changes can be the main reason that modulates the risk of having food allergy.^[3] Recently, the Modern Diet Hypothesis has been raised, proposing that a modern diet is related to increased allergies through promoting an inflammatory state, or introducing immune-toxic compounds and novel allergens to the diet.^[3–4]

Thermal processing is widely used in the modern food industry and home cooking, during which the Maillard reaction (MR) readily occurs between reducing sugars and the amino groups of proteins, peptides, and free amino acids. Meanwhile, the majority of food allergens would undergo heat treatments prior to consumption, and glycation of allergens was shown to impact their biological and

immunological characteristics.^[5] During MR, a large quantity of MR products (MRPs) can be formed, including these glycosylated structures that are covalently conjugated to the proteins, collectively called the advanced glycation end-products (AGEs).^[6] Previous studies have confirmed that the MR reaction could impact the antigenicity/allergenicity of various food allergens *in vitro* and *in vivo* but the results remain controversial. In addition to changes in the structure and accessibility of IgE-binding epitopes, several studies implied that the MR and AGE-modification of food allergens would affect their immunostimulatory capacity and the down-stream T-cell response, which led to inconclusive outcomes in their allergenic manifestations.^[5–10]

Recently, epidemiologic studies suggested that the consumption of foods high in AGEs (e.g., fast foods and fried meat) is associated with a higher incidence of allergic diseases, including food sensitivity.^[8,11,12] The potential link between dietary AGEs and food allergy drives the hypothesis that a diet high in AGEs may consistently prime innate signaling and immune responses,

1. Introduction

Over the past decades, the incidence of food allergy is drastically increased, especially in westernized developed countries.^[1,2]

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inadvertently leading to the misinterpretation of a threat from co-consumed food allergens.^[13] This is based on the fact that, on the one hand, AGE-modification of food allergens is shown to stimulate the uptake of antigens by antigen-presenting cells (e.g., dendritic cells, DCs) and promoted a T helper 2 cells (Th2)-skewed immune response *in vitro*^[7,9]; on the other hand, when absorbed into the circulation, dietary AGEs can interact with the receptor for advanced glycation end-products (RAGE), a transmembrane protein belonging to the immunoglobulin superfamily, and the ligand-RAGE axis with down-stream pathways is evidenced to amplify undesired immune and pro-inflammatory responses.^[14] Specifically, the RAGE is highly expressed on multiple immune cells including DCs, T lymphocytes, and mast cells, and activation of RAGE receptors seems to play a crucial role in the enhancement of adaptive immune responses.^[13] In addition, it is worth noting that several studies reported a putatively harmful effect of dietary AGEs on the homeostasis of gut microbiota (GM), and dysbiosis of the GM is also related to the development of food allergy.^[5,15,16] For instance, a high AGEs diet reduced the abundance of the genus *Lactobacilli* and *Bifidobacteria* in rats,^[17] while their abundance was closely associated with the maintenance of oral tolerance as noted in rodent models.^[18,19]

Thus far, despite previous studies reporting the allergenicity of glycated proteins, there lack investigations that directly evaluate the role of dietary AGEs on the immunoreactivity of typical food allergens. Moreover, in addition to changes induced by allergen-specific AGE-modifications, there exist a large quantify of allergen-non-specific AGEs in the diet, which may also influence the allergenicity of a bystander food allergen through multiple mechanisms, for instance, via induction of a specific GM signature linked to the disease.^[5,13] However, to our knowledge, very little is known about the differences of those two forms of AGEs in the pathogenesis of food allergy, results of which would benefit the understanding of risk factors of allergy in the modern diet.

Considering this, this study aimed to investigate the role of different types of AGEs on the allergenic manifestation of a model food allergen, ovalbumin (OVA) in a BALB/c mouse model. The OVA-specific AGEs were prepared by glycation OVA with a highly reactive AGE precursor, methylglyoxal (MGO) as we earlier reported,^[20] and the OVA-non-specific AGEs were obtained by heating an allergen-free chow diet according to previous studies.^[21,22] The allergic outcomes were measured by targeting key aspects of the immune responses in food allergy, including clinical symptom signs, specific antibody isotypes, lymphocyte subpopulation and related cytokines, injuries in the intestinal barrier, and profiling of the fecal GM. The ability of AGEs to activate RAGE was also evaluated *in vitro* and *in vivo* to provide insights into its role in the sensitization patterns of OVA under different conditions.

2. Results

2.1. Generation of AGEs in OVA-AGE and H-AGE Diet

In this study, to investigate the pathogenic role of dietary AGEs in food allergy, avian OVA was used as a model food allergen and both allergen-specific (i.e., OVA-AGE) and non-specific AGEs (i.e., H-AGE diet) were considered. Firstly, generation of fluorescent and non-fluorescent AGEs in the heated samples was de-

termined and the results are shown in **Figure 1A–C**. After glycation treatment, the fluorescence intensity of OVA-AGE had an approximately 100-fold increase when compared to native OVA, suggesting the formation of a mass of fluorescent AGEs. A 20-fold increase in the fluorescence signal was also witnessed in the H-AGE diet when compared to the intensity of the unheated standard diet. Furthermore, the contents of specific non-fluorescent AGEs, namely, CEL, MG-H1/3, MG-H2, and MOLD were drastically increased in OVA-AGE when compared to native OVA. To a lesser extent, the H-AGE diet contains significantly higher levels of CEL, CML, G-H1, MG-H1/3, and MG-H2, and the content of total AGEs was 2-fold higher than the unheated diet. These results imply that common heat treatment of a food allergen and chow diet both can generate a mass of AGE products, and can be used as the source of allergen-specific and non-specific AGEs co-consumed with the allergen OVA.

2.2. Immunoglobulin E (IgE)-Binding and soluble RAGE (sRAGE)-Binding Abilities of OVA-AGE

Glycation treatment of food allergens is shown to destroy/mask their epitopes, but with the potential to generate health-hazardous AGEs, which are potent ligands of RAGE to activate downstream pro-inflammatory pathways.^[23] The IgE-binding and sRAGE-binding abilities of OVA-AGE were further determined and the results are shown in **Figure 1D,E**. Compared to native OVA, the IgE-binding level of OVA-AGE was reduced by almost 40%, implying that AGE-modification decreased the IgE-binding ability of OVA. However, as shown in **Figure 1E**, the native OVA was almost unable to bind sRAGE, but OVA-AGE displayed an inhibition rate of approximately 85%. These results clearly manifest that, the hypo-antigenic OVA-AGE contains a significant amount of sRAGE ligands, which may influence its behaviors related to immunogenicity and allergenicity *in vivo*.

2.3. Food Allergy Symptoms and Serological Analysis

Subsequently, a BALB/c mouse food allergy model was established by intragastric (i.g.) with cholera toxin (CT) to investigate the allergenicity of OVA in the presence of different forms of AGEs. Previous work has reported that the application of CT, a mucosal adjuvant, alone was not enough to trigger any allergic responses in BALB/c mouse models.^[24,25] BALB/c mice were randomly divided into five groups: the PBS group, OVA group, OVA-AGE group, H-AGE group, and H-OVA group (**Figure 2A**). The first three groups were fed a standard diet of AIN-93G, and the latter two groups were fed an H-AGE diet with identical calorie and macronutrient profiles. Mice in the OVA and H-OVA groups were immunized and challenged by native OVA, and mice in the OVA-AGE group were sensitized and challenged by the same amount of OVA-AGE. The weights of all mice were first recorded, and the results are given in **Figure S1**, Supporting Information. In comparison to the PBS group, on the 31st day, the OVA, OVA-AGE, H-OVA, and H-AGE groups exhibited a significant decrease in body weights ($p < 0.01$). Furthermore, the core temperature of each mouse after the last challenge was shown in **Figure 2B**. Compared to the unimmunized controls (PBS/H-AGE group), mice from the OVA and OVA-AGE groups, but not

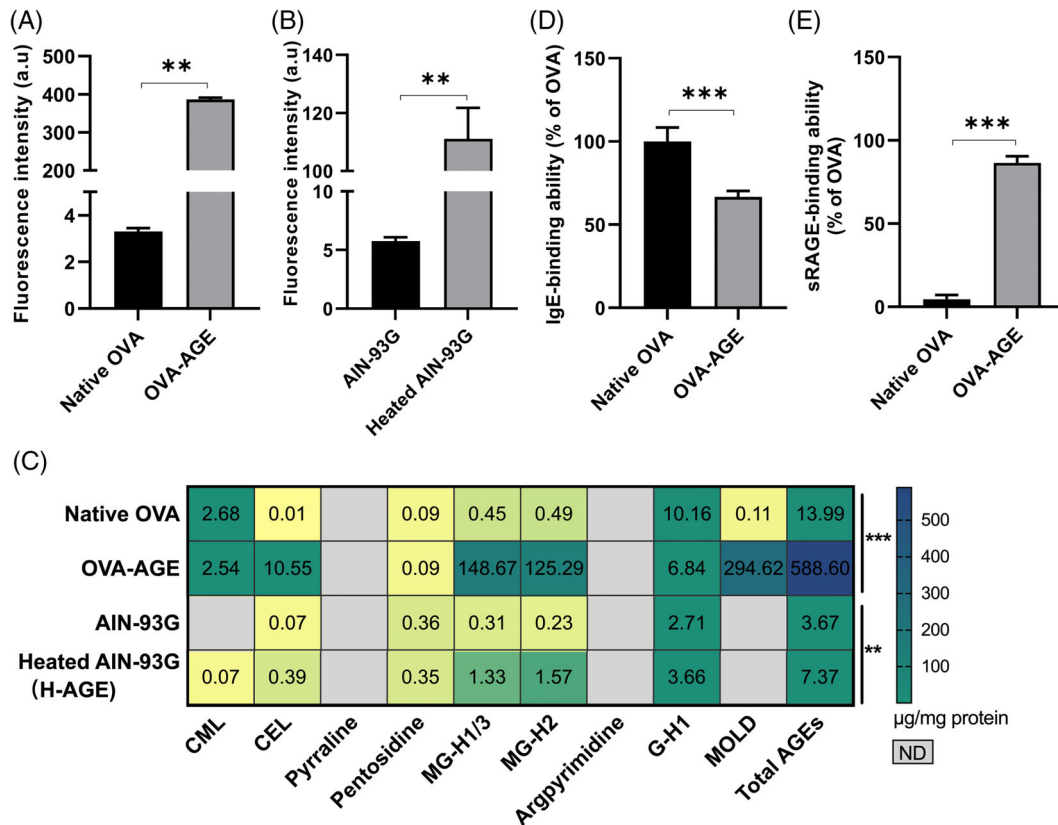


Figure 1. Generation of fluorescent AGEs in A) OVA-AGE and B) H-AGE diet; C) quantification of 10 specific AGEs in the heated samples; D) IgE binding; and E) sRAGE-binding levels of OVA-AGE and OVA in vitro. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the indicated control, student's t -test A, B, E) or Mann–Whitney U test D). ND, not detected.

the H-OVA group, showed a significant reduction in the body core temperature ($p < 0.01$).

Serum levels of OVA-specific antibodies, including IgE, IgG1, and IgG2a, were detected and shown in Figure 2C–E. Compared to the unimmunized groups, mice sensitized with OVA showed increased levels of allergen-specific antibodies, suggesting the successful establishment of food allergy responses in the constructed mouse model ($p < 0.01$). However, despite a near 40% reduction in the IgE-binding ability of OVA-AGE in vitro, we did not see any significant differences in the serum levels of IgE and IgG1 in different sensitized groups. Nonetheless, IgG2a, a Th1-driven antibody was statistically lower in the OVA-AGE group, when compared to the OVA group ($p < 0.05$). Consistently, the IgG1/IgG2a ratio was highest in the OVA-AGE group ($p < 0.05$) (Figure 2F), confirming a predominantly Th2 response induced by glycated OVA in vivo.

2.4. Analysis of Cytokine Production and Immune Cell Subpopulations

In addition to allergen-specific antibodies, the serum levels of related cytokines, including interferon-gamma (IFN- γ), interleukin-4 (IL-4), IL-5, and IL-10 were detected and shown in Figure 3. IL-4 and IL-5 are typical Th2 cell cytokines, and IFN- γ belongs to the Th1 cells.^[26] IL-10, mainly produced by reg-

ulatory T cells (Tregs), monocytes, and mast cells,^[27] is a suppressive cytokine that can regulate excessive immune responses and damage to the body.^[26,27] In line with previous reports,^[25,28] compared to the PBS control, significantly higher levels of IL-4/5/10 mediators were observed in the OVA-induced group ($p < 0.05$), while the content of IFN- γ was marginally changed. However, in comparison to the H-AGE control, the H-OVA group displayed a lower level of IFN- γ ($p < 0.05$) but similar concentrations of the other cytokines. Interestingly, the H-AGE group showed higher levels of serum IL-4 and IL-5 than the PBS control ($p < 0.05$), demonstrating that a high-AGE diet may change the profile of immune responses by modulating the balance between cytokines. The H-OVA group showed a lower production of IL-10 than the OVA group ($p < 0.01$), while no cytokines were differentially secreted between the OVA-AGE and OVA groups ($p > 0.05$).

Further, the subpopulation of splenic lymphocytes was analyzed by flow cytometry, and results are summarized in Figures S2, Supporting Information and 4. Compared to the PBS control, the percentages of cluster of differentiation 4 (CD4)⁺ CD183⁺ Th1 cells and CD4⁺ CD25⁺ Foxp3⁺ Treg cells were significantly decreased, while that of the CD4⁺ ST2⁺ Th2 cells were statistically increased in the OVA group ($p < 0.05$), exhibiting a typical imbalance of T cell subsets in allergic disorders.^[1] Consistently, the H-OVA group had a greater proliferation of Th2 cells with a less abundance in Treg cells than the H-AGE control ($p < 0.05$). Moreover, we noticed a marginally higher Th2

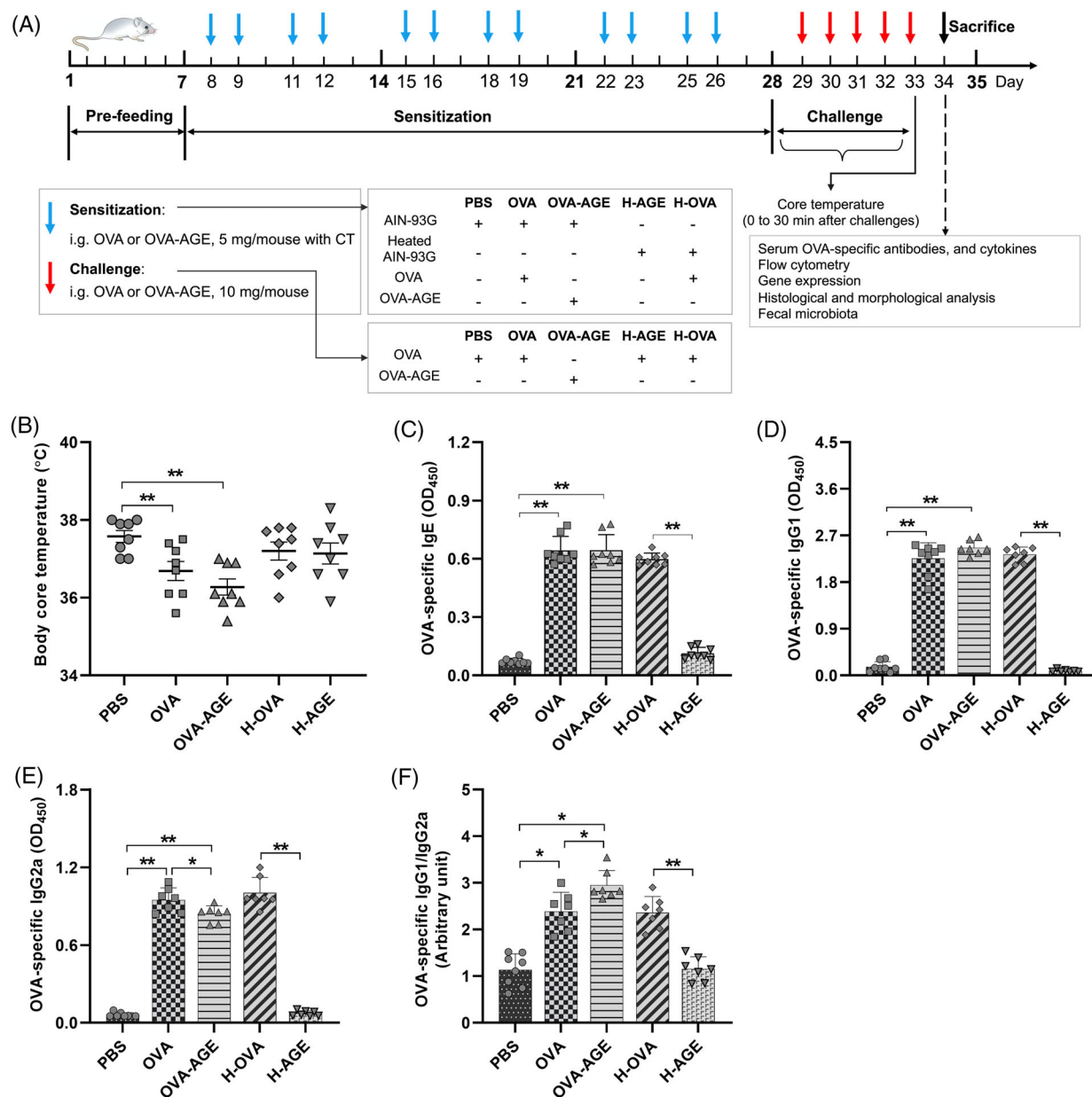


Figure 2. A) The immunization protocol of BALB/c mouse food allergy model; changes in the B) core temperature upon challenge of individual mice ($n = 8$); serum levels of OVA-specific C) IgE, D) IgG1, E) IgG2a, and F) IgG1/IgG2a ratio ($n = 7-8$). * $p < 0.05$, ** $p < 0.01$, compared to the indicated control, Student's t -test B) or Mann-Whitney U test C-F).

subpopulation with a lower Treg subpopulation in the OVA-AGE group than in the OVA group. The DCs maturation phenotype was also measured by targeting costimulatory molecules of CD86 and major histocompatibility complex class (MHC) II via flow cytometry (Figure 4D,E). In comparison to the PBS and H-AGE controls, the sensitized groups witnessed significantly higher expressions of both costimulatory markers ($p < 0.05$). However, inconsistent with the results of T cell subsets, the presence of both forms of AGEs decreased the expression levels of CD86 and MHC II in DCs when compared to the OVA group, especially seen in the H-AGE-induced group ($p < 0.05$).

Further, as RAGE is highly expressed in various immune cells including lymphocytes and DCs, we next determined its mRNA expression level in the spleen of mice in different groups. As given in Figure 4F, compared to the PBS control, the H-AGE group exhibited an up-regulated mRNA level of RAGE in the spleen ($p < 0.05$), suggesting that a high-AGE diet could induce the expression of RAGE in vivo. In the immunized groups, intake of OVA-specific and non-specific AGEs further promoted the mRNA levels of RAGE, when compared to the OVA group ($p < 0.01$). Of note, a high-AGE diet containing non-specific AGEs appears to be more potent to induce the expression of RAGE in vivo.

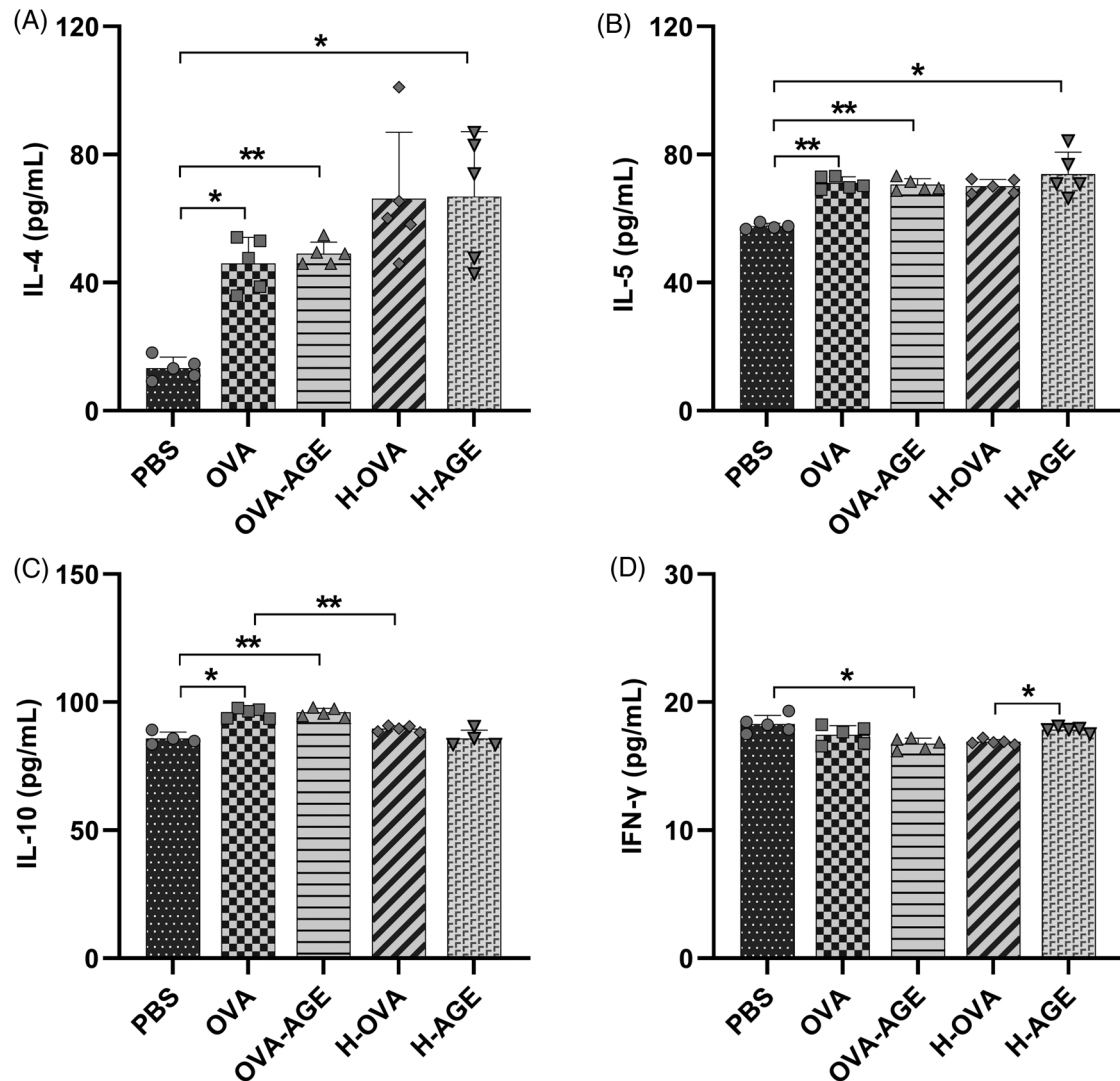


Figure 3. Serum concentrations of cytokines in mice immunized by OVA with different forms of AGEs ($n = 4-5$): A) IL-4; B) IL-5; C) IL-10; D) IFN- γ . * $p < 0.05$, ** $p < 0.01$, compared to the indicated control, student's t -test A) and D) or Mann-Whitney U test B) and C).

2.5. Impacts on the Intestinal Barrier Function

In addition to imbalances in immune cell differentiation and activation, food allergy is often accompanied by injuries in the intestinal epithelium and barrier function.^[29] The integrity of the intestinal barrier was further analyzed by hematoxylin and eosin (H&E) staining of the jejunum tissue and scanning electron microscope (SEM) scan of the jejunal villi of mice in different groups. As shown in Figure 5A,B, compared to the PBS group, the jejunum tissues of mice from the OVA, OVA-AGE, and H-OVA groups exhibited damages to various extents, as evidenced by breaks in the connections, distorted crypts, and even autolysis of the jejunal villi. The H-AGE group also showed a moderate degree of epithelial tissue injury, when compared to the PBS control. Of note, among immunized groups, the H-OVA group had the severest damage in jejunum tissues, which suggested a double action of OVA-challenge and intake of high dietary AGEs on the breakdown of intestinal epithelia.

Based on the histological findings, the expression levels of tight junction (TJ) proteins in the small intestine, including tight junction protein 1 (ZO-1), occludin, and claudin-2 (Figure 5C), were examined by qRT-PCR and the results are given in Figure 5D. As expected, compared to the unimmunized controls, the OVA, OVA-AGE, and H-OVA groups all showed significantly down-regulated mRNA levels of ZO-1, occludin, and claudin-2 ($p < 0.05$). Of note, the H-OVA group displayed the lowest mRNA levels of TJ-related proteins, in line with the results of the histological evaluation (Figure 5A,B). In addition, the H-AGE group also showed down-regulated mRNA levels of claudin-2 and occludin ($p < 0.01$), when compared to the PBS control. To verify the results of gene expression levels, the jejunum tissue was subjected to immunohistochemical (IHC) staining and the results are shown in Figure 5E,F. Similarly, mice in the OVA, OVA-AGE, H-OVA, and H-AGE groups all presented significantly down-regulated protein levels of ZO-1, occludin, and claudin-2 when compared to the mice in the PBS group ($p < 0.01$). Of note, the H-

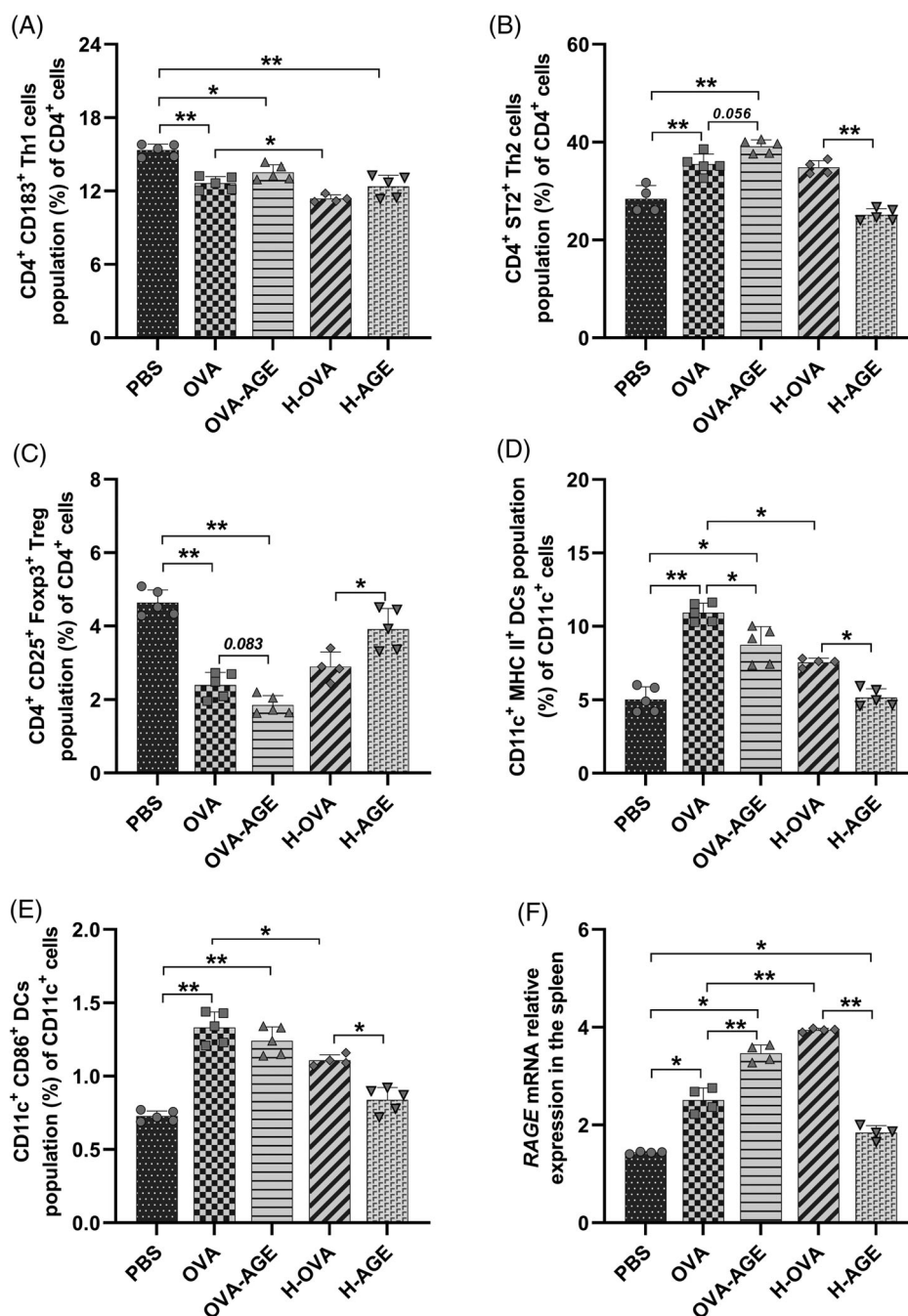


Figure 4. Subpopulations of T lymphocytes and DCs in the spleen of mice immunized by OVA with different forms of AGEs ($n = 4-5$): A) CD4⁺ CD183⁺ Th1 cells; B) CD4⁺ ST2⁺ Th2 cells; C) CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Treg); D) CD11c⁺ MHC II⁺ DCs; E) CD11c⁺ CD86⁺ DCs; and F) relative expression of RAGE mRNA in spleen ($n = 4$). * $p < 0.05$, ** $p < 0.01$, compared to the indicated control, Student's t -test.

OVA group exhibited the lowest protein level of occludin among all groups.

2.6. Changes in the Fecal Microbiota Composition

Since changes in the composition of GM were closely associated with the etiology of food allergy, we finally assessed whether GM

compositional change was involved in the sensitization pattern of OVA with different forms of AGEs. All samples exhibited 100% of good's coverage, suggesting that the analysis of fecal microbiota was based on a good depth sequencing. The α -diversity analysis, i.e., Chao1 and Shannon indices, was firstly used to reflect the abundance and diversity of microbial communities within each group, respectively. As shown in **Figure 6A**, compared to the PBS control, the average Chao1 was significantly higher in the OVA

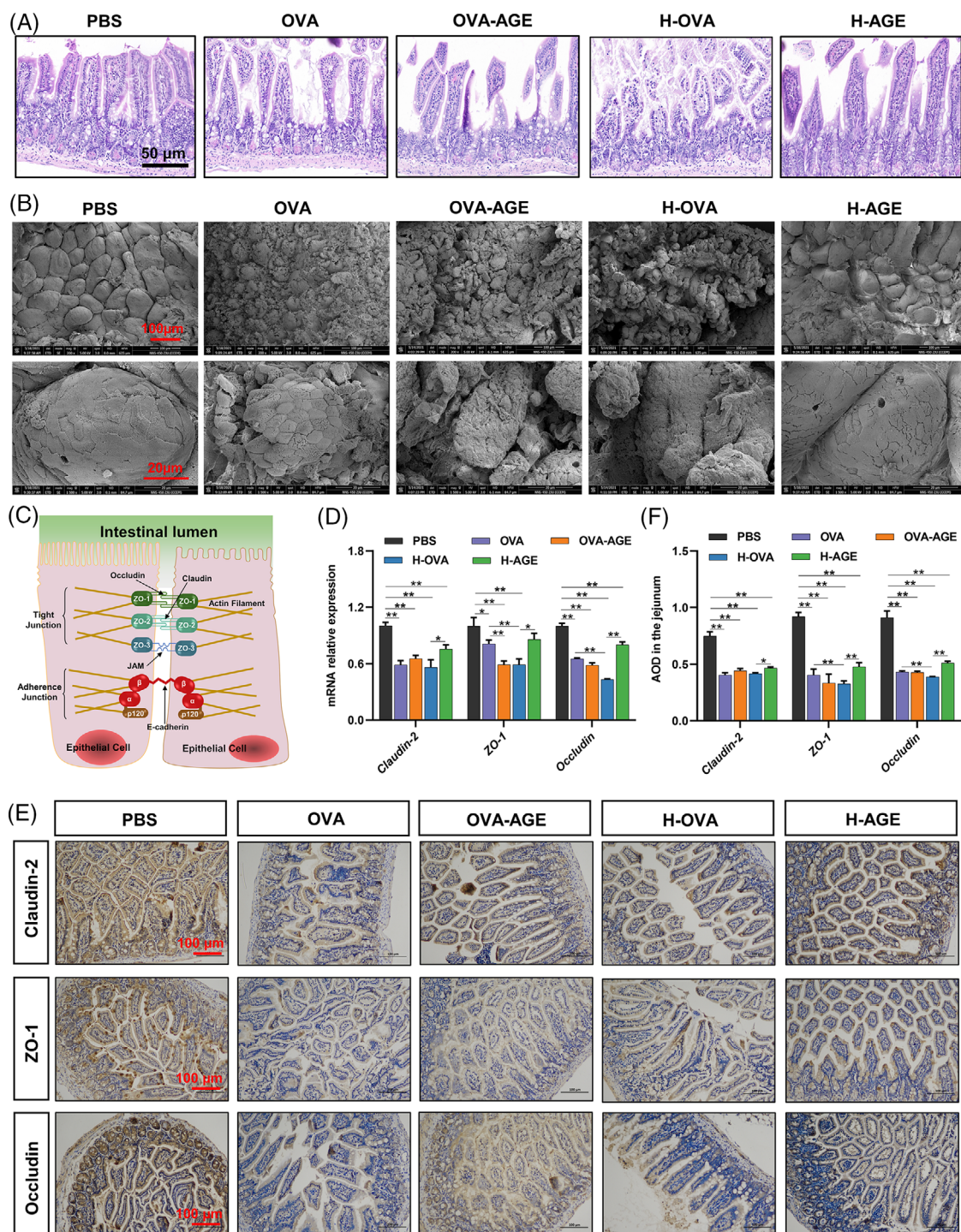


Figure 5. Changes in the intestinal barrier function of mice sensitized by OVA with different forms of AGEs: pathological morphology of jejunum villi as assessed by A) H&E staining (scale bar = 50 μ m, $n = 3$) and B) SEM, (scale bar = 20/100 μ m, $n = 3$); C) a schematic illustration showing different intercellular junctions in the intestinal epithelia, adapted from Dicks et al.⁵⁷⁾; D) the expression levels of genes related to intestinal barrier integrity in the intestine ($n = 4$): claudin-2, ZO-1, and occludin; E) representative images of immunohistochemistry staining for claudin-2, ZO-1, and occludin proteins (scale bar = 100 μ m, $n = 3$); F) average optical density (AOD) changes of claudin-2, ZO-1, and occludin proteins in the jejunum tissue. * $p < 0.05$, ** $p < 0.01$, compared to the indicated control, student's t -test (F and D-claudin-2, ZO-1) or Mann–Whitney U test (D-occludin).

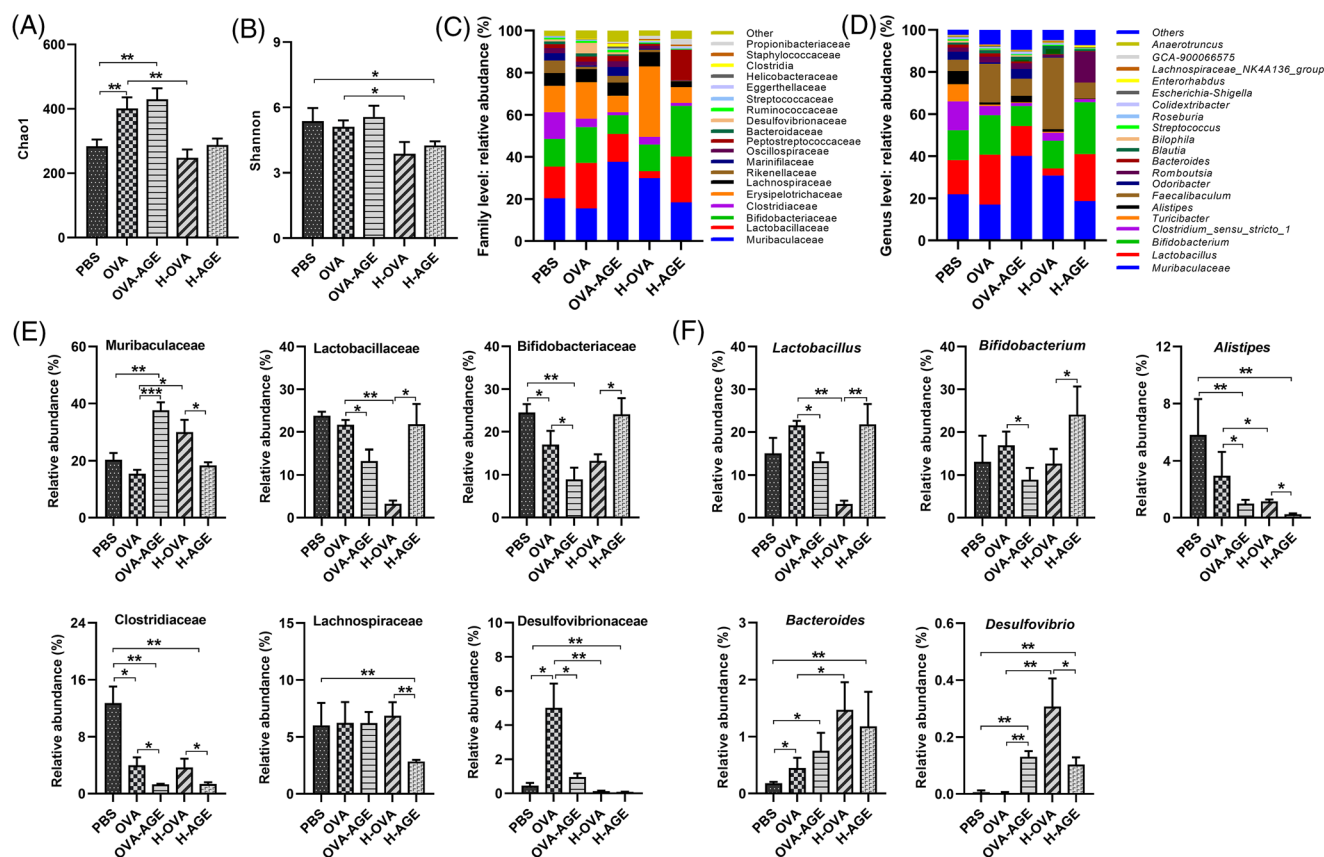


Figure 6. Changes in the α -diversity of fecal microbiota in mice sensitized by OVA with different types of AGEs (n = 5): A) Chao 1 index; B) Shannon index; bacterial relative abundance at the C) family and D) genus levels; and the relative abundance of main bacteria at E) family and F) genus levels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the indicated control, student's t -test (A–F except for E) *Bifidobacteriaceae* and F) *Bifidobacterium* and *Desulfovibrio*) or Mann–Whitney U test (E, *Bifidobacteriaceae* and F, *Bifidobacterium* and *Desulfovibrio*).

and OVA-AGE groups ($p < 0.01$), which implied a greater GM community richness after being sensitized by OVA/OVA-AGE. For the Shannon index, no obvious changes were noticed in the PBS/OVA/OVA-AGE groups; whereas, the H-AGE and H-OVA groups showed a significantly lower Shannon index than the PBS control ($p < 0.05$; Figure 6B), indicating a reduction in GM community diversity after exposure to dietary AGEs.

Further, the relative abundance of bacteria at the family and genus levels in different groups was calculated and shown in Figure 6C,D. The most dominant phylum in all samples was Firmicutes, followed by Bacteroidetes, and Actinobacteriota (Figure S3, Supporting Information). At the family level (Figure 6E), compared to the OVA group, the OVA-AGE and H-OVA groups showed an increased abundance of *Muribaculaceae* ($p < 0.001$) and decreased levels of *Lactobacillaceae* and *Desulfovibrionaceae* ($p < 0.05$), and the OVA-AGE group additionally displayed reductions in the proportions of *Bifidobacteriaceae* and *Clostridiaceae* ($p < 0.05$). Moreover, the H-AGE group witnessed a lower abundance of *Clostridiaceae*, *Lachnospiraceae*, and *Desulfovibrionaceae* than the PBS control ($p < 0.01$). At the genus level (Figure 6F), compared with the OVA group, mice treated with OVA-AGE exhibited a higher abundance of *Desulfovibrio* ($p < 0.01$), but with lower abundances of *Lactobacillus*, *Bifidobacterium*, *Clostridium* ($p < 0.05$), and *Alistipes* ($p < 0.05$); instead, mice in the H-OVA

group had a greater expansion of *Bacteroides* and *Desulfovibrio* with lower proportions of *Lactobacillus* and *Alistipes* ($p < 0.05$). Additionally, the H-AGE group displayed greater proportions of *Bacteroides* and *Desulfovibrio* ($p < 0.01$) with reduced levels of *Clostridium* and *Alistipes* ($p < 0.01$) than the PBS control.

Subsequently, the linear discriminant analysis effect size (LEfSe) analysis was applied and the results are given in Figure 7. In the taxonomic cladogram (Figure 7F), the circles from the inside to the outside refer to the structure of GM from the phylum to the species level, and obvious differences in the taxa can be seen between every two communities. LEfSe results revealed 6, 7, 5, 2, and 12 significant discriminative features in the PBS, OVA, OVA-AGE, H-OVA, and H-AGE groups, respectively (linear discriminant analysis [LDA] score ≥ 4 , Figure 7B). Species richness was highest in the H-OVA group. Compared to the OVA group, *Marinifilaceae*, *Oscillospirales*, and *Odoribacter* were abundant in the OVA-AGE group, and *Faecalibaculum* was abundant in the H-OVA group. Of note, the OVA-AGE and H-OVA groups both possessed a lower abundance of *Desulfovibrio*.

3. Discussion

Heat-processed food is highly consumed in Western diets, which seems to be associated with increased allergies in westernized

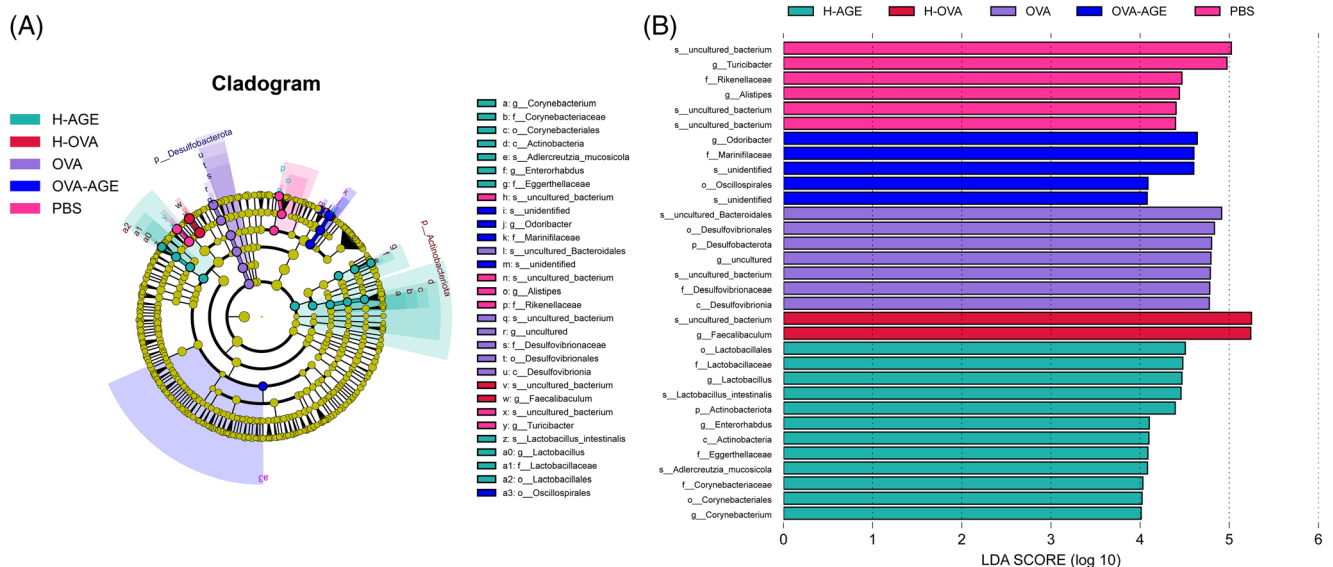


Figure 7. Linear discriminant analysis effect size (LEfSe) analysis showing the species that best explain the differences within groups ($n = 5$): A) taxonomy cladogram; B) Taxa with a linear discriminant analysis (LDA) score greater than 4 are shown.

developed countries. Thermal processing would induce multiple biochemical reactions in foods including the MR, which results in the glycation of food proteins and generation of AGEs. Epidemiologic studies have evidenced that the consumption of H-AGE foods is correlated to the incidence of allergic disorders, including food allergy.^[11,13] In this context, besides epidemiologic observations, how dietary AGEs from different sources impact the sensitization outcomes of common food allergens needs to be investigated via mechanistic approaches. Therefore, this study aimed to investigate the influence of different forms of AGEs on the allergenicity of OVA using both in vitro and in vivo methods targeting key aspects of the immune responses in food allergy. Considering that dietary AGEs co-consumed with OVA can be derived from two sources: allergen-specific AGEs formed in glycosylated OVA, and allergen-non-specific AGEs formed in the diet, these two types of AGEs were induced using simulated heating to prepare the OVA-AGE and H-AGE diet, respectively. Compared to native OVA, the OVA-AGE contains high levels of fluorescent AGEs as well as MGO-related AGEs including CEL, MG-H1/H3, MG-H2, and MOLD. These specific AGEs are also common types of AGEs that are found in Western-style diets.^[30,31] In comparison, the H-AGE diet contained moderately higher levels of CEL, CML, G-H1, MG-H1/H3, and MG-H2, and when calculated based on the food intake ($\approx 5.0 \text{ g day}^{-1}$), a mouse exposed to the heated AIN-93G would ingest 7.35 mg AGEs per day, which is 2-fold higher than the mouse exposed to the unheated diet (data not shown). Despite different types and contents of AGEs in the heated samples, these two products represent typical conditions of consuming a high-AGE diet with the co-ingested allergen OVA. In the following experiments, OVA-AGE was used to analyze the role of glycation and AGE-modification on the allergenicity of OVA, and the H-AGE diet was used to explore the influence of a long-term non-relevant AGEs exposure on the development of OVA-induced food allergy.

It has been reported that MR and glycation may reduce the allergenicity of food allergens via modifying, destroying, and mask-

ing their immunogenic epitopes.^[32] Herein, the IgE-binding ability of OVA-AGE was determined using indirect competitive ELISA. Results showed that, compared to native OVA, the IgE-binding level of OVA-AGE was reduced by about 40%, which can be ascribed to the covalent modification and condensation between AGEs and the amino acid residues of OVA.^[33,34] In our previous report, we demonstrated that glycation of OVA with typical AGE precursors like MGO and glyoxal could reduce the IgE-binding level of OVA by up to 72% when heating at 95°C .^[20] The locations of AGE-modified sites, as revealed by high-resolution mass spectrometry (MS), were found to fall within the sequences of several known epitopes of OVA, thus providing a masking effect on the epitope-sIgE recognition. However, it is worth noting that a lower IgE-binding ability does not guarantee hypo-allergenicity in vivo. In recent years, the MR has long been criticized for the production of health-hazardous AGEs, which are potent ligands of RAGE to activate downstream pathways related to oxidative stress, inflammation, and adaptive immune responses.^[6,13,35] As expected, the OVA-AGE showed a prominently higher sRAGE-binding ability than the native OVA, manifesting that the hypo-antigenic OVA-AGE contained a significant level of RAGE ligands. In this context, previous studies suggested two additional effects of AGE-modification on the allergenic properties of food allergens: on the one hand, the AGE-modified proteins may undergo differential recognition, uptake, and processing patterns by DCs to induce specific down-stream immune responses; on the other hand, the formed AGEs may interact with RAGE that is highly expressed on multiple immune cells like lymphocytes and DCs, and subsequently promote down-stream pro-inflammatory responses and cytokine secretions.^[8-10,13]

Subsequently, a BALB/C mouse model of egg allergy was established to verify the differences in the allergenicity of OVA with different forms of AGEs. Food allergy is defined as an adverse reaction that repetitively occurred after exposure to a given food and is mainly regulated by IgE-mediated responses, including anaphylaxis, decreased body temperature, and severe gastrointesti-

nal reactions. Changes in the body temperature were first used to evaluate the extent of food anaphylactic reactions in mice.^[6,28] Surprisingly, the mice challenged by OVA-AGE and OVA displayed a similar reduction in the body temperature, inconsistent with a near 40% reduction in the IgE-binding level of OVA-AGE in vitro. Of note, mice from the OVA and OVA-AGE groups, but not the H-OVA group, showed a lower body temperature change than the corresponding unimmunized control, manifesting that different types of AGEs may have a differential impact on the allergenic outcomes of OVA in vivo. In previous, Zhang et al. reported that tropomyosin (TM, the main allergen of shrimp) glycosylated by fructo-oligosaccharide triggered severer allergy symptoms in mice than the native TM, ascribed to the presence of neoallergens related to AGE-modification.^[7] In the following sections, systemic immune responses as affected by the allergen-specific and non-specific AGEs will be deciphered from key aspects of the disease.

Further, antibody measurements confirmed that the mice sensitized by OVA with different forms of AGEs harbored a similar level of serum OVA-specific IgE and IgG1. However, we observed a significantly lower level of IgG2a and a higher ratio of IgG1/IgG2a in the OVA-AGE-induced group, when compared to the OVA group. It is known that IgE and IgG1 are Th2-dependent antibodies, while the production of IgG2a relies on the Th1 response.^[36] Therefore, a higher IgG1/IgG2a ratio is indicative of a predominantly Th2 response,^[19] a key driver of IgE-mediated disorders in food allergy. These results highlighted that the OVA-AGE induced a stronger Th2-response than native OVA, which may be ascribed to the presence of AGE-modifications that interacted with the receptors on DCs to induce down-stream Th2-response and B cell activation.

The T cell subpopulations and associated cytokine secretion are important foundations for the induction of food allergic responses. Here we noticed that, despite non-significant differences in the serum levels of Th2-cytokines (i.e., IL-4 and IL-5) in sensitized mice, the OVA-AGE group showed a marginally increased subpopulation of Th2 cells than the OVA group, in consistence with our previous data that OVA-specific AGEs promoted a Th2 polarization in vivo. In addition, the induction of allergen-specific Treg cells is a crucial event to promote oral tolerance. A marginally lower proportion of Treg cells was also noticed in the OVA-AGE group when compared to the OVA group. It is also of interest to see that the OVA-non-specific AGEs induced a lower Th1 cell subset than the OVA group. The distinct T-cell subpopulation may indicate differences in the modulative mechanisms of different forms of AGEs in type 1/type 2 balance. However, since the regulatory cytokines did not complement the results of T cell subpopulations, it is suggestive that, there may exist other pathways involved in the difference between systemic and local responses, such as the role of the innate lymphoid cells and epithelial barrier function linking to local immune responses.^[37,38]

To gain deeper insights into the role of dietary AGEs in OVA-induced allergic response, we further assessed the functional phenotype of antigen-presenting cells (APC, i.e., DCs) in the spleen. Antigen uptake by APC is the first step in adaptive immunity and can be affected by the types and forms of ingested proteins. In addition to antigen uptake, cell maturation plays a vital role in DCs to gain their full T-cell stimulatory capacity, and

the co-stimulatory (CD80, CD86) and MHC class II molecules on the cell surface are considered as makers of the maturation of DCs.^[10] Inconsistent with the results of T cells subpopulation, we noticed lower proportions of CD11c⁺ CD86⁺ DCs and CD11c⁺ MHC II⁺ DCs in the OVA-AGE and H-OVA groups when compared to the OVA group. In previous, Hilmenyuk et al. found that priming mouse bone marrow-derived murine dendritic cells (BMDCs) with glycated OVA did not change the basal expressions of CD80, CD86, and MHC class II molecules in vitro.^[10] Even Rupa et al. reported that mouse splenic DCs treated with mannose-glycated OVA showed a lower expression of CD86⁺ and MHC II when compared to the OVA-primed control cells.^[39]

In addition to immune cell differentiation and maturation, we next questioned whether RAGE activation is involved in the sensitization outcomes of OVA with AGEs. Previous studies have confirmed that RAGE is highly expressed on immune cells including DCs, lymphocytes, and mast cells, and its up-regulation is shown to play a role in pro-inflammatory responses and epithelial activation.^[13] In the present study, mice sensitized by OVA accompanied with AGEs displayed a significantly higher mRNA level of RAGE in the spleen when compared to the OVA group. The H-AGE group also showed an up-regulated expression level of RAGE than the PBS group, indicating that dietary exposure to AGEs unexceptionally activated the gene expression of RAGE. Previously, Hilmenyuk et al. discovered that, compared to OVA, the glucose-glycated OVA induced a higher expression of RAGE and NF- κ B in immature human monocyte-derived DCs in vitro. The glycated OVA-loaded DCs co-cultured with native CD4⁺ cells produced more IL-6 with a stronger Th2 but a weaker Th1 cytokine response, when compared to the OVA-primed system.^[40] In addition, Dumitriu et al. reported that activation of RAGE was necessary for the maturation of human DCs, and was also required for the survival and functional polarization of naive T cells in vitro.^[41] In the present study, as we examined the RAGE mRNA level in the splenocytes instead of in certain isolated cell fractions, it is unclear that which cell subset was responsible for the high expression level of RAGE. Given that the polarization of T cells induced by OVA with AGEs conflicts with the maturation phenotype of DCs in the spleen, it is suggestive that there may be more complicated mechanisms through which RAGE modulates the functions of adaptive immune cells. Considering that AGEs are widely present in the modern diet, and dietary intake of AGEs would unexceptionally up-regulate the expression of RAGE in splenic cells, further research should focus on elucidating the role of RAGE and RAGE-mediated pathways in the modulation of typical immune cells types through mechanistic approaches.

Growing evidence has suggested that the development of food allergy is associated with dysfunctions in the intestinal epithelial barrier.^[28,29] Although the detailed mechanism is not fully understood, several studies have implied that abnormality in the gut barrier function with an increased permeability is involved in the pathogenesis of food allergy.^[42] Rekima et al.^[29] also observed that the expression level of ZO-1, a typical TJ-related protein, was significantly lower in the intestine of an egg-allergic mouse model. In this study, compared to mice in the OVA group, mice challenged with OVA-specific and non-specific AGEs exhibited inflammations in the jejunal tissue, and atrophy and rupture of

the jejunal villi, which was later confirmed by the down-regulated mRNA and protein levels of ZO-1 and occludin in the intestine. It was also of interest to note that the H-AGE group also exhibited lower mRNA and protein expression levels of TJ-related proteins than the PBS group. In previous, Qu et al.^[21] reported that long-term dietary exposure to AGEs changed the structure of colonocytes and suppressed the expression of ZO-1 and occludin in the colon tissue of rats. Previous studies suggested that the activation of RAGE by dietary AGEs in intestinal epithelial cells could stimulate localized production of proinflammatory cytokines and reactive oxygen species, both of which were shown to compromise tight junctions between epithelial cells, thereby disrupting the integrity of the intestinal barrier.^[43,44] A higher intestinal permeability would allow the undigested allergen proteins/peptides and other toxic compounds (e.g., AGEs) to translocate from the lumen into the circulation to prime host immune responses and amplify inflammatory signals in the body. However, the severity of intestinal barrier injury of mice in different groups did not complement the results of allergic symptoms and antibody levels, which can be ascribed to the combined action of some other immunoregulatory mechanisms, such as the mutual modulation between species in the intestinal microflora and dietary AGEs.^[22]

It has been reported that dysbiosis of GM played a causative role in the development of food allergy and other atopic disorders.^[15] Here, we noticed that exposure to non-relevant AGEs, but not OVA-specific AGEs reduced the α -diversity of fecal microbiota in mice sensitized with OVA. Unexceptionally, dietary exposure to a high-AGE diet alone diminished the Shannon index. Consistently, Qu et al. found that intake of dietary AGEs for 8 months reduced the α -diversity and richness of the cecal microbiota in mice.^[22] A lower α -diversity of stool microbiota has been previously associated with atopic eczema at school age but its role in food allergy remains inconclusive.^[16,45,46] Further, through analyzing the composition of GM at the family level, it was noted that mice fed with OVA-specific AGEs exhibited a lower abundance of *Lactobacillaceae* and *Bifidobacteriaceae* than the OVA group. These two types of classic probiotics were also found to be notably decreased in an OVA-allergic mouse model.^[47] Additionally, species of the *Clostridiaceae* family were witnessed to be underrepresented in children with food sensitization.^[48] Here, we noticed that the OVA-AGE group showed a drastic reduction in *Clostridiaceae* when compared to the OVA group; of note, intervention with the high-AGE diet alone even displayed the lowest proportion of *Clostridiaceae* among all treated groups.

At the genus level, consistent with the above results, the two genera of *Lactobacillus* and *Bifidobacterium* were lower in the OVA-AGE group than the OVA group, and the H-OVA group even witnessed a greater reduction in *Lactobacillus* than the OVA-AGE counterparts. Previous reports have confirmed a lower abundance of *Lactobacillus* in β -lactoglobulin-allergic mice.^[49] Additionally, probiotic supplementation with *Lactobacillus* and *Bifidobacterium* species was shown to be a potential treatment for cow's milk allergy in randomized controlled trials.^[50,51] Moreover, Kirjavainen et al. found that taxa within *Bacteroidetes* were up-regulated in the GM of infants with severer milk allergy, early-onset atopic eczema, and a strong family history of allergic disorders.^[52] Here, we observed a higher abundance of

Bacteroides in mice from the H-OVA group when compared to the OVA group. Furthermore, several studies have highlighted the depletion of the genera *Clostridium* in food-allergic infants and children, especially for those with persistent allergic conditions.^[48] It was also demonstrated that therapy with *Clostridiales* species suppressed food-allergic responses in mice via a Treg cell-dependent pathway.^[53] Here, we observed that mice in the OVA-AGE group suffered a greater depletion of the genera *Clostridium* than the OVA group. Finally, species of the *Alistipes* are considered probiotic and are found to be absent from individuals with *Clostridium difficile* infection, and the *Desulfovibrio* is reported to release a large quantity of hydrogen sulfide that can destroy epithelial intestinal cell integrity and cause barrier dysfunction in mouse models.^[21,54] Here, again, we observed a more significant abundance alteration of those two genera in the OVA-AGE and H-OVA groups, when compared to the OVA-induced counterparts. Overall, these results clearly manifested that both the OVA-specific and non-specific AGEs disturbed commensal microbiota homeostasis to a larger content than the allergen OVA alone, especially seen in the H-OVA group. This stronger effect of the non-specific AGEs to disrupt GM may be ascribed to the higher and consistent intake of various types of AGEs present in the chow diet. It is also worth noting that, exposure to a high-AGE diet alone was able to modify the GM composition, as evidenced by the depletion of some probiotic and allergy-protective genera (e.g., *Clostridium* and *Alistipes*), and the expansion of certain pro-inflammatory communities (e.g., *Desulfovibrio*). This is indicative of a GM-reshaping capacity of heat-processed foods containing dietary AGEs, which may adversely affect the gut and immune health and induce adverse consequences upon a long-term exposure. Further studies should employ in vitro fermentation experiments and germ-free mouse models to explore the role of GM composition as affected by certain types of dietary AGEs in the manipulation of food allergic responses.

We acknowledge this study exists several limitations. Firstly, we characterized the allergic responses of mice fed with two forms of AGEs via targeting systemic and distant-tissue indexes, such as serum cytokines and splenic lymphocyte subsets. To enforce the stringency of the present study, immune responses in local tissues, such as lamina propria, Payer patch, or mesenteric lymph nodes should be explored for studying the role of allergen-specific and non-specific AGEs on the allergenic manifestation of OVA. Secondly, the sample number of some experiments, such as SEM, H&E, and IHC, was small and warrant deeper validation in future mechanistic experiments.

In conclusion, this study, for the first time, systematically compared the impact of two typical sources of dietary AGEs on the allergic outcomes of OVA in vivo, from several key aspects of the disease etiology. It was observed that these two forms of AGEs influenced the sensitization pattern of OVA via different mechanisms: the OVA-specific AGEs are more prone to induce a Th2-biased immune response, while the OVA-non-specific AGEs are more potent to disrupt the intestinal barrier, induce RAGE up-regulation and GM dysbiosis. This can be ascribed to the allergen-dependent and independent pathways as mediated by different forms of dietary AGEs, which may include but not limited to: 1) the innate signaling regulated by different AGEs and their receptors; 2) the interactions between innate and adaptive immunity; and 3) the crosstalk between microbiome and host immune sys-

tem. As we did notice some conflicting results of the immune cell phenotype and cytokine responses in the present work, mechanistic researches on the biological relevance of certain types of dietary AGEs on the allergen-specific immune responses are warranted in the future.

4. Experimental Section

Materials: OVA ($\geq 98\%$, cat# A5503) and MGO (40% aqueous solution, cat# M0252) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AGEs standards and isotopically labeled internal standards: N ϵ -carboxyethyllysine (CEL, $\geq 95\%$, cat# 25333) and N ϵ -carboxymethyllysine (CML, $\geq 95\%$, cat# 16483) were purchased from Cayman Chemical (MI, USA); methylglyoxal-hydroimidazolone isomers [MG-H1 acetate salt ($\geq 96\%$, cat# HAA3003), MG-H2 acetic acid salt ($\geq 94\%$, cat# HAA3320), MG-H3 trifluoroacetic acid (TFA) salt ($\geq 99\%$, cat# HAA3330)], glyoxal-hydroimidazolone isomers (G-H1, $\geq 98\%$, cat# HAA2970), pentosidine ($\geq 99\%$, cat# HAA3030), pyrrolidine ($\geq 99\%$, cat# HAA3040), argpyrimidine TFA salt ($\geq 99\%$, cat# HAA3050), methylglyoxal-lysine dimer (MOLD acetate salt, $\geq 95\%$, cat# HAA3080), CML-d4 ($\geq 99\%$, cat# HAA2952), CEL-d4 ($\geq 95\%$, cat# HAA2941), MG-H1-d3 acetate salt ($\geq 98\%$, cat# HAA3002), G-H1-13C2 ($\geq 98\%$, cat# HAA2971), and GOLD-15N2 acetic acid salt ($\geq 94\%$, cat# HAA3071) were products from Iris Biotech GmnH (Marktredwitz, Germany). A mouse anti-claudin-2 antibody (cat# ab53032) and horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (cat# ab6789) were bought from Abcam (Cambridge, UK). Human sRAGE recombinantly produced in *E. coli* was purchased from Biovendor (Brno, Czech Republic; cat# RD172116100), and the mouse anti-human RAGE antibody (cat# MAB11451) was a product of R&D systems (MN, USA). The mouse anti-ZO1 (cat# GB111981), anti-occludin (cat# GB111401), and HRP conjugated goat anti-rabbit IgG (cat# GB23303) antibodies were purchased from Servicebio Technology Co., Ltd. (Wuhan, China). Ultrapure (UP) water used in this study was purified from a Millipore Milli-Q Advantage A10 Water Purification System (Bedford, MA, USA). All other chemicals and reagents used were of analytical grade unless otherwise noted.

Preparation of OVA-Specific and Non-Specific AGEs: OVA as the model allergen was used in the present study and further glycosylated by MGO, a typical AGE precursor, to obtain OVA-AGE conjugate according to Heilmann et al.,^[10] with slight modifications. In brief, MGO was mixed with OVA solution with an MGO: lysine residue ratio of 2:1, and the mixture was incubated at 65 °C for 30 min to mimic common pasteurization.^[55] Then, the solution was dialyzed against UP water for 48 h to remove unreacted MGO and salts. The sample was freeze-dried and stored at -20 °C until further analysis (labeled as OVA-AGE, representing allergen-specific AGEs). The allergen non-specific AGEs were produced according to the previous report of Qu et al.^[22] Briefly, the standard AIN-93G diet was heated at 175 °C for 45 min to prepare the high-AGE diet (labeled as H-AGE). Both diets were manufactured by Xietong Bioengineering Co., Ltd. (Nanjing, China), and as shown in Table S1, Supporting Information the two diets were free of allergen and identical in calories and macronutrients level. Generation of fluorescent AGEs in the OVA-AGE or H-AGE diet was determined by fluorometry as described by the previous report,^[56] and the contents of 10 specific AGEs, namely, CML, CEL, MG-H1/3, MG-H2, G-H1, pyrrolidine, pentosidine, argpyrimidine, and MOLD were quantified by an isotope dilution-based UHPLC-MS/MS method as indicated by Zhang et al.^[20]

In Vitro IgE-Binding Ability of OVA-AGE: The IgE-binding ability of OVA-AGE was analyzed by an indirect competitive ELISA assay according to the protocol reported elsewhere.^[20] In brief, the OVA solution (0.5 mg mL⁻¹) dissolved in 0.05 M carbonate buffer solution (pH 9.6) was coated on a 96-well plate (2 μ g protein per well) and then kept overnight at 4 °C. After blocking with 3% bovine serum albumin (BSA) solution, the plate was co-incubated with 100 μ L of the mouse serum (1:100 diluted in PBS) collected in Animal Experimental Section (OVA group) and 100 μ L of OVA-AGE solution (2 mg mL⁻¹) at 37 °C for 1 h. Afterward, 100 μ L of

HRP goat anti-mouse IgE antibody (1:2500) was added to the plate and further incubated for 1 h at 37 °C. Finally, the plate was developed with 50 μ L of TMB substrate reagent in the dark for 15 min at 37 °C and terminated with 50 μ L of 2 M H₂SO₄ solution. The absorbance of the plate was recorded at 450 nm by a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA). Between each step, the plate was washed three times, 5 min each with 0.05% Tween 20 in PBS (PBST). The ability of OVA-AGE to bind OVA-specific IgEs was calculated using the equation:

$$\text{IgE binding ability(\%)} = \left[1 - \frac{A_t - A_b}{A_p - A_b} \right] \times 100 \quad (1)$$

where, A_t , A_p , and A_b represented the absorbance of the treatment group, positive control (without inhibitors), and blank control (serum only), respectively. The final results were expressed as a percentage relative to the IgE-binding ability of native OVA.

sRAGE-Binding Assay: The ability of OVA-AGE to bind sRAGE was measured by a competitive ELISA-based method according to the previous report,^[20] with minor modifications. The specificity was detected by establishing competition between a known high-affinity ligand (BSA-AGE) coated on the plate and the possible ligand formed in OVA-AGE. Briefly, BSA-AGE solution (1 mg mL⁻¹) diluted in 1.5 mM carbonate buffer solution (pH 9.6) was coated on a 96-well plate (100 μ L per well) and then kept overnight at 4 °C. The plate was washed and blocked with 3% BSA in PBST for 2 h at room temperature. Meanwhile, a mixed solution was prepared by incubating OVA-AGE (1 mg mL⁻¹) with sRAGE (27.4 nM) in 1.5% BSA/PBST for 45 min at 37 °C. After blocking, the plate was washed and incubated with the mixed solution (100 μ L per well) for 1 h at 37 °C. Then, the plate was washed and added with mouse anti-RAGE body (1 μ g mL⁻¹) for another 1 h at 37 °C. After repeatedly washing, the plate was incubated with the detection antibody (HRP goat anti-mouse antibody, 1:5000) for an additional 1 h at 37 °C. Following repeated washing, 50 μ L of TMB substrate reagent was added for color development and the reaction was terminated by 50 μ L of 2 M H₂SO₄. The absorbance was read at 450 nm with 620 nm as a reference by a microplate reader. The inhibition rate was calculated with the following equation:

$$\text{Inhibition rate (\%)} = \left[1 - \frac{A_s}{A_R} \right] \times 100 \quad (2)$$

where, A_R and A_S represented the absorbance of the wells containing sample, and the wells containing only sRAGE but without any competitors, respectively.

Experimental Design and Animals: BALB/c mice (female, 6-week-old) were purchased and housed in the Laboratory Animal Research Center of Hangzhou Normal University (Zhejiang, China). Protocol was approved by the Institutional Animal Care and Use Committee of Animal Laboratory Center of Hangzhou Normal University and the Zhejiang Gongshang University Ethics Review Committee (2021032001). The housed environmental conditions were controlled as follows: 22 $\pm$ 1 °C temperature, 55 $\pm$ 10% relative humidity, and a 12-12 h light-dark cycle. The mice were randomly divided into five groups (n = 8 per group), i.e., the PBS control group, OVA group, OVA-AGE group, H-OVA group, and H-AGE group. Mice in the PBS/OVA/OVA-AGE group were fed a standard AIN-93G diet, and mice in the H-OVA/H-AGE group were fed an H-AGE diet (i.e., the heated AIN-93G diet containing high levels of AGEs). Figure 2A presents the treatment, immunization, and sampling protocol. In brief, after 1-week acclimation, each mouse in the immunized group was sensitized via intragastric (i.g.) gavage of 0.2 mL PBS containing 5 mg OVA (for OVA/H-OVA group) or OVA-AGE (for OVA-AGE group) with 10 μ g adjuvant of CT (Sigma-Aldrich, St. Louis, MO, USA) four times a week for 3 weeks. Mice in the PBS control and H-AGE groups were administered intragastrically with 0.2 mL PBS containing 10 μ g CT instead. In the 5th week, the mice were challenged by i.g. gavage of 10 mg OVA or OVA-AGE for 5 consecutive days (day 29–33). After the last challenge (day 33), the core temperature of each mouse was determined within 30 min, and 24 h later, the mice were anesthetized and killed by cervical dislocation. The blood was collected

and serum prepared, and the spleen and jejunum tissues were harvested under sterile conditions. Fresh feces were also collected and snap-frozen with liquid nitrogen before storage at -80°C .

Serum OVA-Specific Antibodies, and Cytokines Level: The serum levels of OVA-specific antibodies, including IgE, IgG1, and IgG2a were measured by indirect ELISA as previously reported.^[28] Moreover, the levels of IL-4, IL-5, IL-10, and IFN- γ in the sera were detected by commercial ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA), per the manufacturer's instructions.

Flow Cytometry: Following the last challenge and sacrifice, the spleen tissues were obtained and prepared for single-cell suspensions. The cells were washed, suspended in sterile PBS, and probed with anti-CD4-FITC, anti-CD183-APC, and anti-ST2-APC antibodies for Th1/Th2 analysis, or anti-CD4-FITC, anti-CD25-PE, and anti-Foxp3-APC antibodies for Treg analysis. For DC maturation analysis, the cells were stained with anti-CD11c-FITC, anti-MHC-II-PB450, and anti-CD86-PE antibodies. All antibodies were purchased from Biolegend (San Diego, CA, USA). If required, cells were fixed and permeabilized with BD fixation/permeabilization reagent (BD bioscience, Franklin lake, NJ, USA). All samples were detected with a CytoFLEX Flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), and the data were analyzed by CytExpert software (Version 2.0, Beckman Coulter, Inc., Brea, CA, USA).

Analysis of Gene Expression by Real-Time Quantitative PCR (qRT-PCR): The RNA of the spleen and jejunum tissues was isolated and extracted by a Total RNA Extraction Kit (Solarbio Science & Technology Co., Ltd., Beijing, China), and the RNA content was detected with a NANO DROP 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and then adjusted to the same level. Then 5 μg of total RNA was used to synthesize the cDNA by the Hifair III cDNA Synthesis SuperMix qPCR kit (Yeasten Bio-Technologies Co., Ltd., Shanghai, China), according to the manufacturer's protocol. Specific primers (Table S2, Supporting Information) were designed and synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China). The qRT-PCR analysis was performed using the Hieff UNICON Universal Blue SYBR qRT-PCR kit (Yeasten Bio-Technologies Co., Ltd., Shanghai, China) with a LightCycler 480 II qRT-PCR system (Roche, Switzerland). Data were normalized to the housekeeping genes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed with the $\Delta\Delta$ cycle threshold method.

Histological and Morphological Analyses of Jejunum Tissue Section: After the last challenge and sacrifice, the jejunum tissue samples were collected and fixed in 4% paraformaldehyde buffer for 24 h. The samples were dehydrated, embedded in paraffin, sectioned at a thickness of 4 μm , and finally stained with H&E in a blinded fashion for histopathological analysis. The results were analyzed with Caseviewer software (3DHISTECH Ltd., Hungary). The integrity of the jejunum villi was observed by SEM, according to a previous report.^[28]

Immunohistochemistry: IHC staining was conducted on the above jejunum tissue sections by using primary antibodies of anti-ZO1 (1:500), anti-occludin (1:300), and anti-claudin-2 (1:300), and secondary antibody of HRP conjugated goat anti-rabbit IgG (1:200). The sections of IHC were pictured using a light microscope and quantified by Image J software (version 1.53q, National Institutes of Health, MD, USA). The results were shown as the average optical density (AOD).

Fecal Microbiota: 16S rRNA Sequencing: Stool samples from the mice after the last oral challenge were collected and snap-frozen with liquid nitrogen before storage at -80°C . The DNA extraction and amplification of 16S rRNA including the hypervariable region (V3–V4) were performed on an Illumina NovaSeq 6000 platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). A common primer pair was used in the present study. The Quantitative Insights into Microbial Ecology 2 (QIIME2) software was used for analysis of the bioinformatics of sequencing data in this study. Data processing and sequence analysis were completed by the Novogene Company (Beijing, China) by using standard protocols unless otherwise specified.

Statistical and Bioinformatic Analysis: All experiments were completed in at least triplicates unless otherwise noted, and the results were reported as the mean $\pm$ standard deviation (SD). GraphPad Prism (version 8.3.1; GraphPad Software, San Diego, CA, USA) and SPSS Statistics software (version 25.0; SPSS Inc., Chicago, IL, USA) were used for statistical analysis.

Differences between groups were determined by one-way analysis of variance (ANOVA, followed by Duncan's multiple range test), Student's *t*-test or Mann–Whitney *U* test, where $p < 0.05$ was considered significant.

For microbial profile analysis, the α -diversity analysis (i.e., Chao1 and Shannon indices) and relative abundance (%) of bacteria at the genera/phylum level were generated by using QIIME2 software. The significant differences in phylotypes among groups were identified by a Taxonomy-based analysis. Based on the Mann–Whitney *U* test, only the taxa with an average abundance $>0.1\%$, p value <0.05 , and q value (risk of false discovery) <0.1 were marked significant. The linear discriminant analysis effect size (LEfSe) was used to analyze the biomarkers that best explain the microbial differences between indicated groups, and the threshold for the LDA score was set at 4.0.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

G.Y. and Q.Z. contributed equally to this work. Y.G. contributed to experimental conduction, data analysis, and manuscript writing. Z.Q. involved in study design, data integration, and manuscript revision. L.H. contributed to AGEs quantification of the samples. W.Y. involved in the conceptualization and experimental supervision. F.L. contributed to funding acquisition, conceptualization, and manuscript revision.

Keywords

advanced glycation end-products, food allergy, gut microbiota, immune response, intestinal barrier function

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