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ORIGINAL ARTICLE

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Development of a low allergenic product for patients with milk allergy and assessment of its specific IgE reactivity

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Abstract

Background: Milk oral immunotherapy is the riskiest and most unpredictable form of oral immunotherapy. We aimed to produce a low allergenic product than conventional once baked-cake/muffin, to develop indirect in-house ELISA to check the tolerance status with milk products and evaluate IgE reactivity of patients' sera via western blotting (WB) and indirect in-house ELISA.

Method: A low allergenic product named biscotti-twice baked-cake was developed, and the total protein concentration was determined. The protein content was studied by SDS-PAGE and proteomics. Milk-specific IgE (sIgE) binding assays were performed by WB and indirect in-house ELISA by using patients' sera.

Results: Casein band intensity was observed to be lower in the biscotti-twice baked-cake than in the once baked-cake ($p = .014$). Proteomics analysis and αS1-casein measurement showed that the lowest intensity of casein was found in biscotti. The low binding capacity of milk sIgE to biscotti compared with once baked-cake was shown by WB ($p = .0012$) and by indirect in-house ELISA ($p = .0001$). In the ROC analysis, the area under the curve (AUC) of the in-house ELISA IgE was comparable with Uni-CAP milk and casein sIgE. The AUC of the in-house ELISA IgE for cake (0.96) and biscotti (1) was slightly better than Uni-CAP milk sIgE (0.94; 0.97) and casein sIgE (0.96; 0.97), respectively.

Conclusion: The low allergenicity of the newly developed low allergenic product "biscotti-twice baked-cake" has been demonstrated by in vitro experiments. Biscotti could be a safe treatment option than once baked-cake/muffin in patients who are reactive to once baked-milk products.

KEYWORDS
baked milk, casein, cow's milk allergy, ELISA, food allergy, IgE reactivity, immunotherapy, low allergenic food product, proteomics

1 | INTRODUCTION

Cow's milk protein allergy (CMPA) is one of the most common food allergies (FAs) in children, constituting 40% of the FAs followed in pediatric allergy clinics.[1] The prevalence is estimated to be 3.8% in children under 5 years of age. The severity of the reaction and the degree of sensitivity depend on the amount of milk consumed, the type of preparation, milk protein-specific (s) IgE levels, and the

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phenotype of the patient. Cow's milk (CM) ranks third both in food-induced anaphylaxis (10%–19%), and in fatal or near-fatal food-induced anaphylaxis (8%–15%).[2–4]

CM contains approximately 30–35 g/L of CM protein. Major CM allergens are casein, β-lactoglobulin (β-LG), and α-lactalbumin.[5] Cooking or flavoring processes (heat, fermentation, pasteurization, matrix effect, and Maillard reaction) may change the allergenicity of the proteins; thus, the frequency and severity of the allergic reactions can be reduced or increased.[6,7] Casein and α-lactalbumin are more heat resistant than whey proteins (β-LG, bovine serum albumin).[5] Boiling the milk at 120°C for 15 min does not impair the antigenic properties of casein proteins, which has linear epitopes so protein chains that break down into smaller pieces when denatured by heat maintain their epitope property and continue to bind to IgE.[5,8] However, β-LG has conformational epitopes and loses its ability to bind IgE when denatured with boiling heat. Thus, exposure to high (200°C) and prolonged (30 min) temperatures, such as baking, leads to the destruction of conformational epitopes and deterioration of the three-dimensional structure of allergens, thereby reducing the allergenicity of the proteins including β-LG and caseins.[9]

Another effect that contributes to baked food tolerance is the matrix effect.[10] It is suggested that protein, fat, and sugar interactions that make up the matrix reduce IgE binding by closing allergen epitopes. Moreover, if proteins are exposed to heat in the presence of sugar and water, a reaction called glycation (Maillard reaction) occurs, which modifies the structure and the aggregation/allergenic effects of milk proteins in different ways depending on the different combinations of protein, sugar, and temperature.[6,11] Glycation gives the nutrition brown color, flavor, and aroma and stands for the breakdown of proteins without enzymes; therefore, it also disrupts epitopes by degrading the proteins. Patients with CMPA reactive to baked milk products constitute the distinct phenotype group, who are more probably reactive to casein rather than β-LG. More crucially, compared with a strict avoidance diet, which is currently the "gold standard of care," adding baked milk products to the diet appears to accelerate the development of tolerance to unheated milk.[12–15]

The objectives of this study were to develop a new low allergenic food product than the conventional once baked-cake that contains milk proteins with low reaction risk in patients who are even reactive to once baked-cake and to evaluate reliable indirect in-house ELISA for the detection of sIgE antibodies against low allergenic baked milk product.

2 | METHODS

The development and production of biscotti-twice baked-cake is shown in Figure 1A. The detailed descriptions of methods and anaphylaxis diagnosis are included in Appendix S1.

Key message

Although oral immunotherapy with baked milk seems safe, anaphylaxis may still occur in some patients. The newly developed low-allergenic product "biscotti-twice baked-cake" could be a safer treatment option than cake/muffin in patients who are reactive to once baked-milk products.

3 | RESULTS

Fifty-seven patients (42.1% female, median age: 4.09 [IQR: 1.74–5.88] years) with CMPA were included in this study. Thirty patients (52.6%) were additionally allergic to other foods (e.g., egg and lentil). Thirty-eight of them (66.7%) had a history of anaphylaxis when exposed to milk products (Table S1).

3.1 | Total protein concentration and casein band intensity in biscotti 3 h was lower than the conventional cake

CM proteins were run in SDS-PAGE and stained to visualize the protein content (Figure 1B). The protein bands in Figure 1B showed the casein content of CM (albumin, caseins, β-LG, and α-lactalbumin).[16]

Milk protein concentrations from five different milk products (CM, conventional milk once baked-cake, biscotti-twice baked cake 1, 2, and 3 h) were shown in quintuplicate in Table S2. The protein concentration of the biscotti-twice baked-cake 3 h was significantly lower than the once baked-cake (Figure 1C). Based on these concentrations, the same amount of protein was used in the following experiments. Noticeably, the casein band intensity decreased as the cooking time increased for biscotti-twice baked-cake samples (Figure 1D) and were significantly lower than the CM and once baked-cake, respectively (Figure 1E).

3.2 | Proteomics results showed that the intensities of intact milk proteins decrease as the cooking time increases

The proteomics analysis by LC-qTOF-MS showed that the intensities of each casein fraction and β-LG were decreased as the second baking time increased. The reduction in intensities was found to be statistically significant for each casein fraction and β-LG (Figure 2A). The lowest level of intact protein was always observed in biscotti-twice baked-cake 3 h. The results of αs1-casein ELISA showed that αs1-casein concentration level in biscotti-twice baked-cake 3 h samples was found significantly lower than once baked-cake (Figure 2B).

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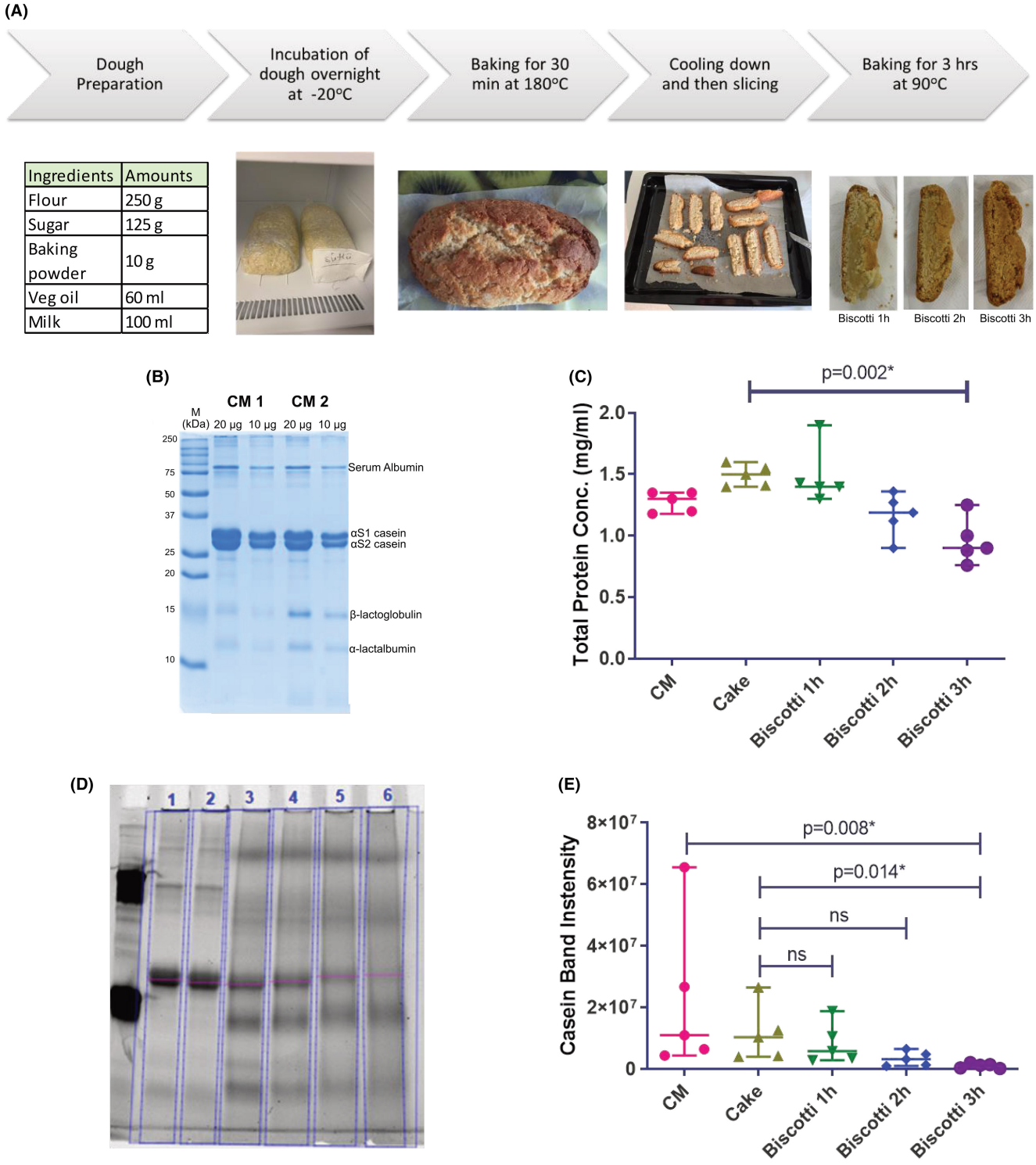


FIGURE 1 Development and production of biscotti and protein quantitation analysis; (A) Two steps of baking: First step is the classical/conventional baking of the overnight frozen cake at 180°C, 30 min. Second step is the additional baking of the cake slices at 90°C for 3 h to have biscotti product. (B) Commassie staining of SDS-PAGE for the proteins of cow's milk (CM1, first cow's milk sample; CM2, second cow's milk sample; M, marker) (C) Comparison between the total protein concentrations of different milk products measured by the Bradford Assay ($n = 5$). (D, E) Casein band intensity analysis of milk, cake and biscotti samples done with Image Lab, Biorad ($n = 5$, 1: Cow's milk, 2: Baked Cow's milk, 3: Cake, 4: Biscotti 1 h, 5: Biscotti 2 h, 6: Biscotti 3 h). *Comparison of cake and biscotti-3 h; comparison of cow's milk and biscotti-3 h by nonparametric post hoc analysis.

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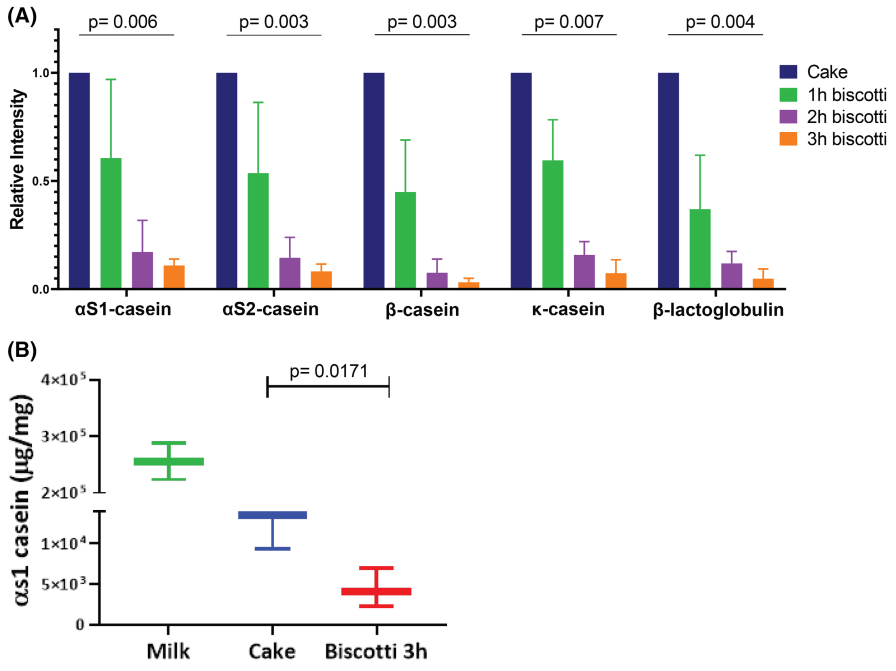


FIGURE 2 Detailed examination of protein contents of cake and biscotti. (A) LC-qTOF-MS analysis of the cake and biscotti samples ($n = 5$, Biscotti samples were normalized to milk. Analysis was done with the Related-Samples Friedman's Two-Way Analysis of Variance by Ranks) (B) αs1 casein concentration was evaluated by commercial ELISA kit for milk, cake and biscotti 3 h ($n = 3$).

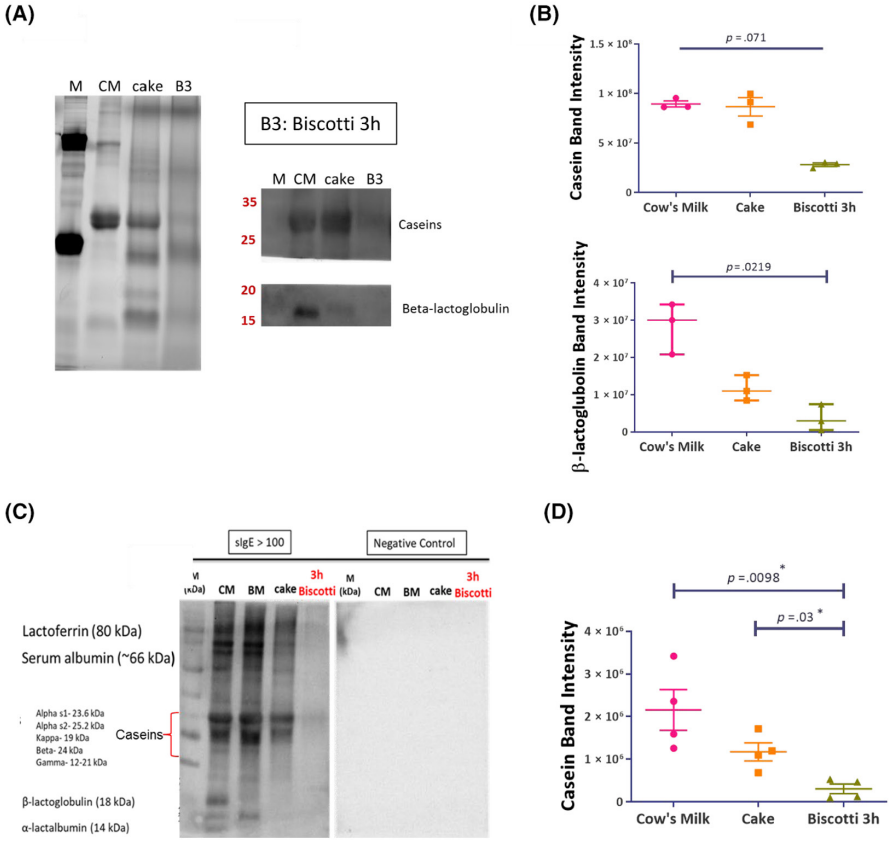


FIGURE 3 Binding intensity of specific IgE to caseins and β-lactoglobulin by western blot in biscotti, conventional cake (baked milk) and pasteurized milk samples. (A) SDS-PAGE image of the pasteurized milk, cake and Biscotti 3 h (B3) samples and PVDF membrane incubated with anti-casein and anti-beta-lactoglobulin antibodies ($n = 3$), (B) Casein and β-lactoglobulin band analysis of milk, cake, and biscotti 3 h with Image Lab ($n = 3$), (C) PVDF membrane incubated with three patients' sera with sIgE higher than 100 kU/L, and negative control ($n = 4$), (D) Casein band analysis of milk, cake, and biscotti 3 h with Image Lab ($n = 4$), *cake and biscotti-3 h, cow's milk, and biscotti nonparametric post hoc analysis, CM, Pasteurized cow's milk; B3, Biscotti 3 h; M, Marker.

3.3 | Milk sIgE of the milk allergic patients bound incompetently to casein bands of biscotti-twice baked-cake

To confirm whether the proteins located between 25 and 37 kDa were the caseins and the ones seen at ~18 kDa were β-LGs, WB was done (Figure 3A). The results revealed that the aforementioned proteins were caseins and β-LGs (Figure 3B).

Moreover, serum of the patients who have CM sIgE greater than 100 kU/L were used to analyze the binding capacity of CM sIgE to milk proteins obtained from the pasteurized CM, once baked-cake, and biscotti-twice baked-cake 3 h (Figure 3C). Milk

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sIgE binding to caseins was significantly lower in biscotti-twice baked-cake 3 hours than once baked-cake and pasteurized CM ($n = 4$, Figure 3D).

3.4 | Lower OD values were obtained with biscotti-twice baked-cake 3 h compared with the once baked-cake

To investigate the reactivity of the milk-allergic patients' sera with once baked-cake and biscotti-twice baked-cake 3 h, indirect in-house ELISA was conducted. The serum dilution of 1:20 was used for all samples according to the checkerboard titrations of the cake and biscotti 3 h samples shown in Figure 4A. The binding of sIgE from 57 CM-allergic patients was significantly lower in biscotti-twice baked-cake 3 h than in once baked-cake (Figure 4B).

3.5 | Lower IgE OD values and CM sIgE levels were obtained with tolerant CM-allergic patients

To investigate the reactivity of the CM-allergic patients' sera with milk, cheese, and yogurt, indirect in-house ELISA was conducted. The OD values and tolerance status of the milk, cheese, and yogurt and the sIgE levels of 35 patients were listed in Table S3.

After determining the tolerance status of patients (reactive or tolerant, Table S3), OD values of the reactive and tolerant ones were compared with each other for milk, cheese, yogurt, once baked-cake, and biscotti-twice baked-cake 3 h, separately (Figure 5A). The in-house ELISA OD values of tolerant patients were lower for milk, cheese, yogurt, once baked-cake, and biscotti-twice baked-cake when compared to reactive ones. The CM sIgE levels (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA) were also lower in milk, cheese, yogurt, once baked-cake, and biscotti-twice baked-cake than the reactive ones (Figure 5B).

(A)

	CAKE							BISCOTTI					
	no sec	1:10.000	1:5000	1:2000	1:1000	1:500		no sec	1:10.000	1:5000	1:2000	1:1000	1:500
no primary	0.048	0.045	0.047	0.046	0.047	0.048	no primary	0.048	0.049	0.045	0.045	0.046	0.049
1:500	0.097	0.104	0.120	0.150	0.228	0.239	1:500	0.091	0.097	0.106	0.132	0.189	0.211
1:250	0.141	0.152	0.179	0.227	0.387	0.405	1:250	0.115	0.138	0.153	0.194	0.308	0.349
1:100	0.225	0.265	0.302	0.427	0.794	0.841	1:100	0.194	0.235	0.271	0.355	0.625	0.721
1:20	0.352	0.392	0.478	0.662	1.257	1.385	1:20	0.298	0.378	0.425	0.582	0.986	1.119
1:10	0.557	0.650	0.742	1.029	1.880	2.126	1:10	0.493	0.605	0.686	0.890	1.495	1.734

(B)

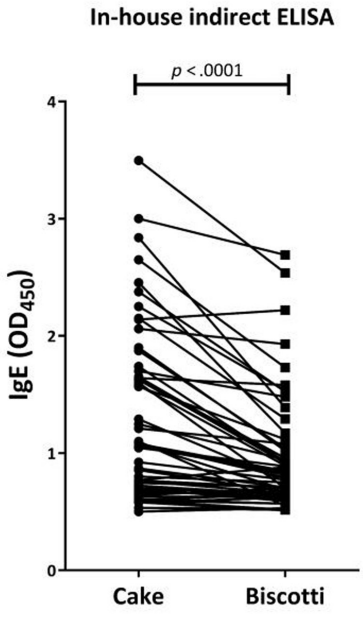


FIGURE 4 Binding intensity of specific IgE to whole milk proteins by in-house indirect ELISA in biscotti and conventional cake (baked milk) samples; (A) Checkerboard titration analysis to determine optimal dilutions done with the patients' sera whose milk sIgE levels >100 kU/L and anti-IgE secondary antibody for both conventional cake and biscotti samples ($n = 3$). (B) The cake/biscotti 3 h specific IgE in-house indirect ELISA results of 57 milk allergic patients, Wilcoxon matched-pairs signed-rank test.

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ROC analysis was performed to determine the diagnostic accuracy of in-house ELISA sIgE and Uni-CAP CM sIgE and casein sIgE tests for tolerance status (Figure 6). While all AUC values were increased from "milk tolerant vs. reactive" to "biscotti tolerant vs. reactive" group, the best AUC values were obtained with "biscotti tolerant vs. reactive" group for both tests. In-house ELISA IgE had lower AUC in milk, cheese, and yogurt than in Uni-CAP milk and casein sIgE; however, in-house ELISA IgE showed higher AUCs for once baked-cake and biscotti-twice baked-cake.

4 | DISCUSSION

This study aimed to develop a new low allergenic food product that contains low allergenic CM proteins for severe CM-allergic patients who are even reactive to conventional once baked-cake and to assess in-house ELISA to check the IgE reactivity of newly developed low allergenic food product, named biscotti-twice baked-cake, in vitro. The casein band intensities were low in biscotti sample even though the same amount of protein was loaded in the gels. Proteomics analysis showed that the intensities of each casein fraction and β-LG were decreased as the baking time of biscotti increased. The low binding capacity of milk sIgE to biscotti compared with the once baked-cake in the sera of children with CMPA was shown by WB and indirect in-house ELISA. Lastly, the diagnostic accuracy of in-house ELISA IgE and Uni-CAP milk sIgE for tolerance status were comparable while AUC of in-house ELISA sIgE for once baked-cake and biscotti-twice baked-cake reactive vs. tolerant was greater than Uni-CAP milk sIgE.

Bloom et al. mentioned that temperature and duration are two important factors in protein allergenicity, along with the presence of wheat and sugar.[17] They showed strongly staining casein bands with gel electrophoresis that persisted for up to 60 min of heating while β-LG and α-lactalbumin bands became progressively weaker with increasing heating times. Several studies showed that 70%–80% of children with CMPA were reactive to pasteurized CM but may tolerate conventional baked milk products (matrix with wheat, sugar, and baked at 180–200°C for 30 min, examples are muffin, cake).[12,18–20] Tolerance to baked milk products in children with IgE-mediated CMPA is a good predictor of milk tolerance development.[12,18,19,21] For the baked milk reactive ones, there is currently no low allergenic choice than a once baked milk product (cake/muffin) to promote desensitization via the oral route.

Total protein concentration was found low for biscotti-twice baked cake. For this reason, all experiments were performed with equal amount of total protein. Due to the Maillard reaction, matrix effect, and high temperature, the concentrations of total protein, the intensities of different protein fractions shown by proteomics, and the concentration of α-S1 casein were low in biscotti 3 h. Significant quantities of Bos d 11 posed a risk for adverse reactions even after the muffin was baked, based on a recent study, because the inside part of the muffin has higher levels of allergens.[22] Biscotti-twice baked-cake formula with the second baking period for 3 h at 90°C provides a homogenous and equally distributed low allergenicity in all compartments of the product, including outer and inner parts. Since the biscotti is firm to eat, after powdering it manually in mortar, the powder form of biscotti became more homogenous and easier to weight for small amounts. Eight non-allergic adults and 17 milk allergic children tasted the biscotti-twice baked-cake, and all of them enjoyed the odor and taste; 13 out of 17 milk-allergic children continued to eat the increasing doses of biscotti powder at home

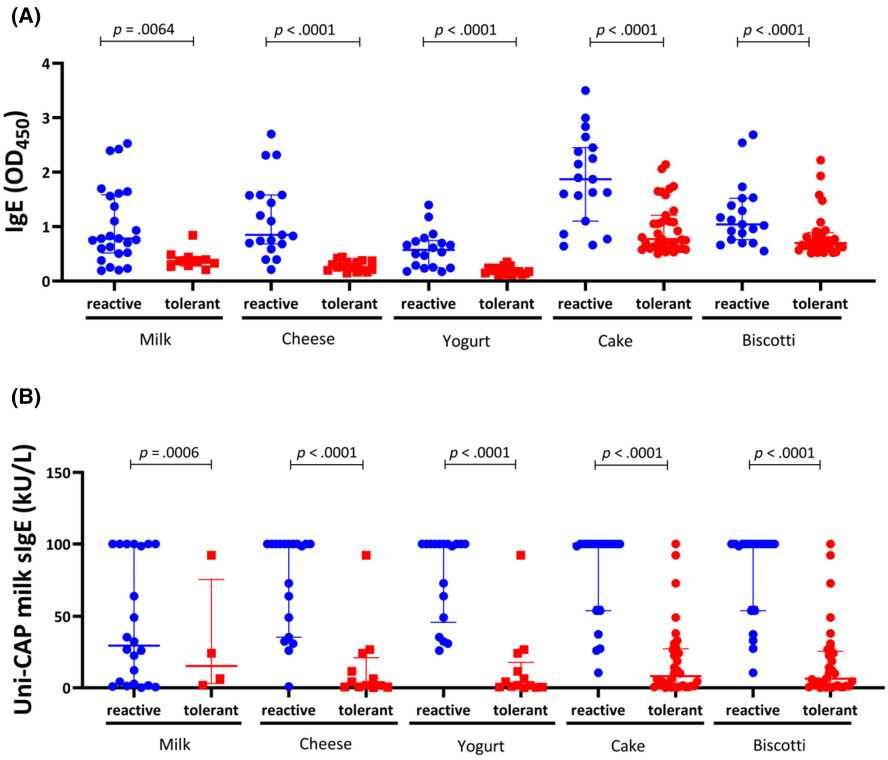
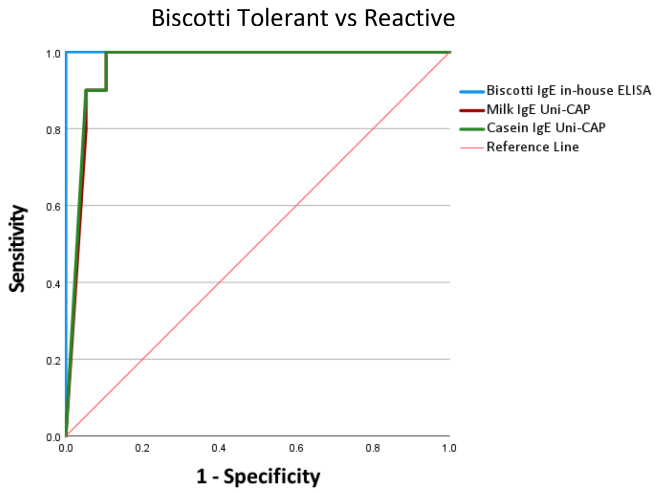
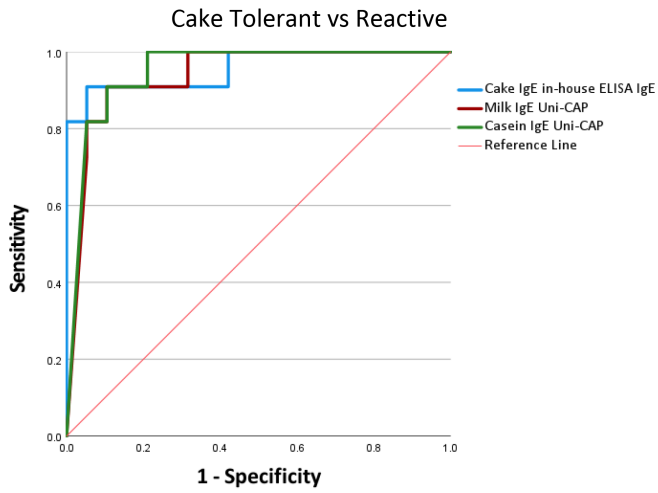
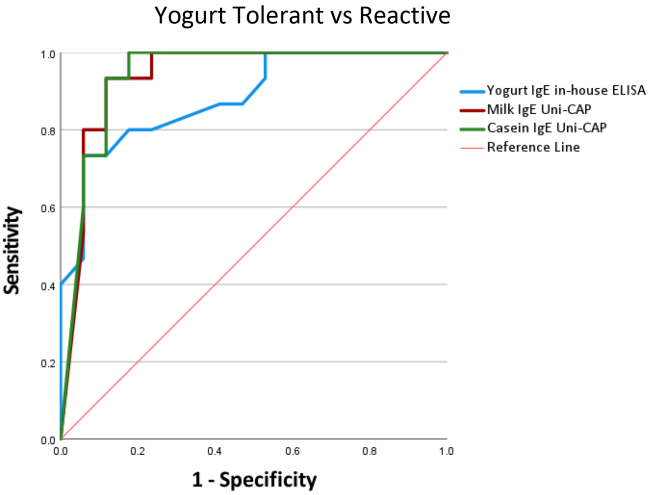


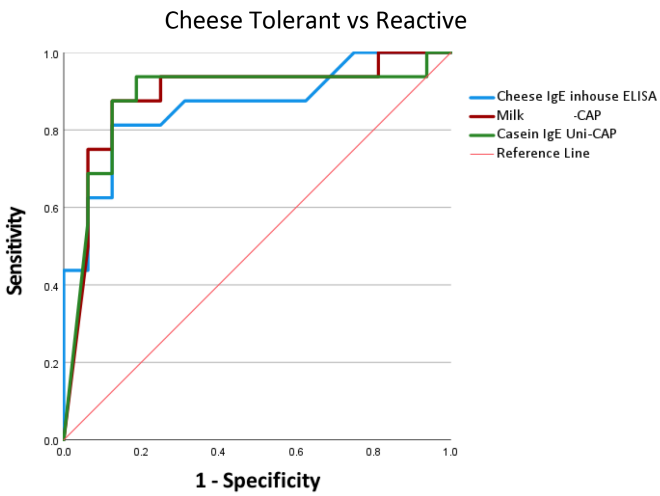
FIGURE 5 Milk, cheese, yogurt, cake, and biscotti sIgE in-house ELISA results (A, milk reactive $n = 21$, milk tolerant = 10, cheese reactive $n = 13$, cheese tolerant = 18, yogurt reactive $n = 15$, yogurt tolerant = 16, cake reactive = 19, cake tolerant = 35, biscotti reactive = 19, biscotti tolerant = 34. All analysis were performed with nonparametric Mann–Whitney *U* test) and Milk sIgE (Uni-CAP, Phadia, Thermo Fisher Scientific, MA, USA) results of milk, cheese, yogurt, cake, and biscotti reactive and tolerant ones (B, milk reactive $n = 19$, milk tolerant = 4, cheese reactive $n = 19$, cheese tolerant = 12, yogurt reactive $n = 18$, yogurt tolerant = 13, cake reactive = 19, cake tolerant = 34, biscotti reactive = 19, biscotti tolerant = 33. All analysis were performed with nonparametric Mann–Whitney *U* test).

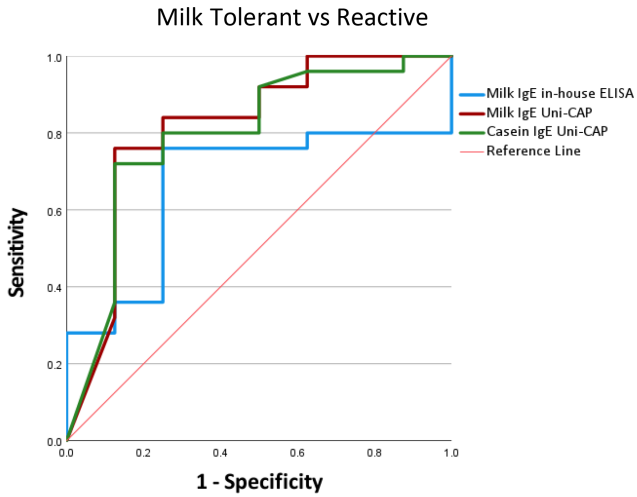
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Milk sIgE	AUC	Sensitivity	Specificity
milk tolerant vs reactive	0.815	0.840	0.750
cheese tolerant vs reactive	0.887	0.875	0.812
yogurt tolerant vs reactive	0.937	0.933	0.882
cake tolerant vs reactive	0.938	0.909	0.895
biscotti tolerant vs reactive	0.963	0.900	0.947
Casein sIgE	**AUC**	**Sensitivity**	**Specificity**
milk tolerant vs reactive	0.795	0.800	0.750
cheese tolerant vs reactive	0.881	0.938	0.882
yogurt tolerant vs reactive	0.939	0.933	0.882
cake tolerant vs reactive	0.950	0.909	0.895
biscotti tolerant vs reactive	0.966	0.900	0.947
In-house ELISA sIgE	**AUC**	**Sensitivity**	**Specificity**
milk tolerant vs reactive	0.665	0.760	0.750
cheese tolerant vs reactive	0.861	0.813	0.882
yogurt tolerant vs reactive	0.882	0.800	0.824
cake tolerant vs reactive	0.957	0.909	0.947
biscotti tolerant vs reactive	1.000	1.000	1.000

FIGURE 6 Diagnostic performance of IgE assays in predicting tolerance outcomes, AUC, area under curve.

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(Table S4). The planned amounts/portions of biscotti powder were separately prepared for each day for a duration of 1 month in small cups, and the parents and children were happy with biscotti powder service too.

To check the IgE reactivity of CM-allergic patients, WB and indirect in-house ELISA were performed in. The mechanism that leads to IgE-mediated allergy depends on the IgE affinities and the epitopes that antibodies specifically bind. WB is the most frequently used method for checking IgE reactivity. Bloom et al. showed that the binding of sIgE to the allergens varied based on the temperature, baking duration, and the presence of wheat by doing WB.[17] Our results demonstrate that more reactivity is observed with CM and then cake[17] and lastly with biscotti.

ELISA is evaluated to detect serum sIgE levels of the total milk and/or milk fractions (casein, β-LG, and α-lactalbumin) for diagnostic purposes. There is a need for more specific ELISA systems for detecting sIgE reactivities against baked milk products (partially denaturated milk proteins found in once baked-cake and biscotti-twice baked-cake samples) as well as fermented products such as cheese and yogurt. Results similar to WB are found in indirect in-house ELISA (for once baked-cake and biscotti) as well. Biscotti indirect in-house ELISA gives lower OD values than the cake. Milk, cheese, and yogurt ELISA results demonstrate that tolerant patients have lower OD values than the reactive ones for every three groups of patients (milk, cheese, and yogurt). Moreover, ROC curve analysis shows that the diagnostic performances of both in-house ELISA IgE and milk sIgE (Uni-CAP) are comparable, and both are useful methods to evaluate the tolerance status. In addition, Hawi et al.[23] found that Uni-CAP assay for milk sIgE had better performance. Moreover, our results showed that the in-house ELISA sIgE for cake and biscotti sIgE had a higher AUC than Uni-CAP milk sIgE. The diagnostic value of casein sIgE in differentiating once baked milk reactive and tolerant patients has been evaluated in several studies. In milk-allergic patients, Caubet et al.[24] showed that a cutoff value of casein sIgE 5 k/UL is a good predictor of determining patients who could tolerate baked milk with a sensitivity of 74% and specificity of 89%. In that study, the authors recommended avoidance of CM products in patients with a casein sIgE higher than 20.2 kU/L since they were more likely to react to baked milk. In addition to component resolved diagnosis giving the opportunity of measuring separately casein and β-LG sIgE levels, we need more useful diagnostic tests determining sIgE levels for once baked-cake, biscotti-twice baked cake, cheese, and yogurt proteins. The in-house ELISA sIgE method, which we developed, may be a new proceeding for clinical practice to distinguish different grades of CMPA patients.

The current study has certain limitations. First, IgE reactivity assays were performed using total protein concentrations. For all milk products, the concentration of caseins should be measured specifically, and calculations would be done according to it. Further studies are required to perform the casein specific assays. Second, it is thought that the Maillard reaction is the reason for the inside and outside brown color of the biscotti. The Maillard reaction may be confirmed by looking the reaction end products via metabolomics.[25] Further experiments should be done to examine the end products of the Maillard reaction. Third, to develop a standard method of sIgE against biscotti and once baked-cake proteins, in-house ELISA experiments should be done in a higher number of patients with different milk allergy phenotypes.

In conclusion, a new low allergenic product called biscotti-twice baked-cake has been developed, which has the potential to be a lower allergenic option than the current once baked-cake. The low casein intensity of biscotti was shown by proteomics analysis. Indirect in-house ELISA is developed and can be used for further experiments to check the IgE reactivity of patients' sera with milk, cheese, yogurt, once baked-cake, and biscotti-twice baked-cake. Biscotti-twice baked-cake displays low IgE reactivity with patients' sera. Lastly, the good diagnostic performance of in-house ELISA IgE is evaluated with ROC curve analysis for tolerance status.

AUTHOR CONTRIBUTIONS

Duygu Yazici: Conceptualization; methodology; investigation; writing – original draft; writing – review and editing. **Hande Suer:** Methodology. **Cemre Naz Bulbuloglu:** Investigation; visualization. **Elif Guzar:** Methodology. **Engin Koçak:** Methodology. **Emirhan Nemutlu:** Methodology; supervision. **Betul Buyuktiryaki:** Conceptualization; writing – review and editing; investigation; supervision. **Cansin Sackesen:** Conceptualization; methodology; writing – review and editing; funding acquisition; project administration; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in relation to this work.

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