Check for updates

Accepted Article

DR JAMES P HINDLEY (Orcid ID : 0000-0002-3262-2897)

DR MARTIN D CHAPMAN (Orcid ID : 0000-0002-0845-3632)

Article type : Original Article-Allergens

Bos d 11 in baked milk poses a risk for adverse reactions in milk-allergic patients.

Running title: Bos d 11 levels remain high in baked milk

Word count: 3893. Table count: 1. Figure count: 5.

James P Hindley PhD[1], Maria A Oliver PhD[1], Cathy Thorpe BS[2], Angharad Cullinane BSc[1], Sabina Wuenschmann PhD[2], Martin D Chapman PhD[2]

[1] Indoor Biotechnologies Ltd, Cardiff, UK

[2] Indoor Biotechnologies Inc, Charlottesville, VA, USA

Corresponding Author:

Martin D. Chapman PhD

Indoor Biotechnologies Inc.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/CEA.13774

This article is protected by copyright. All rights reserved

700 Harris Street

Charlottesville, VA 22903

USA

E-mail: mdc@inbio.com

Tel +1 434 984 2304

Acknowledgements: The authors are grateful to Izabel Alvares for baking the muffins and for additional technical support; and to Bryan Smith and Kristina Reid Black for assistance with the ELISA validation.

Conflict of Interest: All authors are employees of Indoor Biotechnologies and Martin D. Chapman is an owner of Indoor Biotechnologies.

Declaration of Funding: This work was funded in part by a grant from the European Union 7th Framework, Project No. FP7-KBBE-2012-6-312147, and by Indoor Biotechnologies.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement: The concept behind this work was developed by MC. The experimental work was supervised and directed by MC, JH, and MO. Allergen assays of muffin extracts were performed by AC. CT conducted the IgE antibody testing under the supervision of SW. CT and MO prepared the data for publication, including statistical analysis (MO). MC, JH and MO wrote the paper.

This article is protected by copyright. All rights reserved

Accepted Article

Accepted Article

ABSTRACT

Background: Patients are commonly challenged with foods containing baked milk, for example muffins, yet little is known about the specific allergen content of muffins used in milk challenges or of the effect that baking has on allergenicity.

Objective: Our objective was to compare the levels of major milk allergens in uncooked and baked muffins using monoclonal immunoassays and IgE antibody binding before and after baking.

Methods: Uncooked and baked muffins were prepared using recipes from Mount Sinai and Imperial College. Allergen levels were compared by ELISA for Bos d 5 (β-lactoglobulin) and Bos d 11 (β-casein). IgE reactivity was assessed using sera from milk-sensitised donors in direct binding and inhibition ELISA.

Results: Bos d 5 was reduced from 680µg/g in uncooked muffin mix to 0.17µg/g in baked muffins, representing a >99% decrease after baking. Conversely, Bos d 11 levels in baked muffin remained high and only decreased by 30% from a mean of 4249µg/g in uncooked muffin mix to 2961µg/g when baked (~181mg Bos d 11 per muffin). Baked muffins retained ~70% of the IgE binding to uncooked muffin mix. Baked muffin extract inhibited IgE binding to uncooked muffin mix by up to 80%, demonstrating retention of *in vitro* IgE reactivity.

Conclusions & Clinical Relevance: High levels of Bos d 11 in baked muffins pose a risk for adverse reactions, especially in patients who have high anti-casein IgE antibodies.

This article is protected by copyright. All rights reserved

Key Words: Adverse Reactions, Allergen Measurement, Baked Milk, Milk Allergy, Oral Food Challenges

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
LLOD	Lower Limit of Detection
mAb	Monoclonal antibody
NIST	National Institute for Standards and Technology
NHS	National Health Service of the United Kingdom
OFC	Oral food challenge (s)
PBS	Phosphate buffered saline, pH 7.4
SD	Standard deviation

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Introduction

Cow's milk allergy is the most common form of food allergy, affecting around 1-4% of young children in Europe and other Western countries[1-3]. Milk allergy is caused by an adverse immune reaction to one or more of the proteins in cow's milk. Approximately 50% of these reactions are IgE mediated and, in the most severe cases, are associated with anaphylaxis. Although it is arguable whether the prevalence of childhood milk allergy has increased in recent years, there is some evidence that allergic reactions to cow's milk have become more persistent[4;5]. Most milk-allergic children (80%) out-grow the condition by the time of school age and develop clinical tolerance to cow's milk allergens. However, some children have more severe phenotypes associated with high IgE levels and IgE antibodies to casein and may not develop tolerance until adolescence or, in the most severe cases, into adulthood[6]. The patterns of sensitization to individual cow's milk proteins varies significantly by study population and age[6;7]. Approximately 61% of cow's milk reactive individuals are sensitised to Bos d 5 (β-lactoglobulin) and approximately 57% are sensitised to Bos d 11 (β-casein). These proteins represent two of the major allergens in milk[8] and constitute ~9% and ~26%, respectively of total milk protein[9].

Up until 10 years ago, avoidance of milk was the standard of care for management of milk allergy[1]. However, avoidance of milk can be difficult to maintain and can impact the patient's quality of life and nutritional intake[10]. The observation that children who consumed extensively heated (baked) milk became tolerant to milk changed the paradigm for treatment of milk allergy[11]. Consumption of baked milk products accelerated the development of tolerance to milk and was associated with reduced IgE antibody responses to milk allergens[5]. Approximately 70-75% of children treated with baked milk could tolerate baked milk and, ultimately, unheated milk. The rationale for using baked milk was that baking denatured conformational epitopes on milk allergens. Children who could not tolerate baked milk (20-30%) were reported to have higher levels of total and milk-specific IgE antibodies. These children were also reported to have higher IgE antibodies to sequential (or linear) epitopes on milk allergens[12;13].

This article is protected by copyright. All rights reserved

Confirmation of allergy to milk can help to ensure that only those who genuinely need to avoid milk do so. This is best achieved using oral food challenges (OFC) which are integral to the evaluation of food allergy[14;15]. It is common clinical practice to conduct an OFC in patients with suspected milk allergy. Often these challenges are performed using food preparations containing baked milk. Allergists use baked milk materials for OFC under the assumption that they have reduced allergenicity[11;14;15]. However, severe reactions can occur using OFC with baked milk[16;17].

The effects of baking on specific milk allergen proteins have not been fully investigated. There is surprisingly little information about the levels of residual allergen or the IgE reactivity of baked milk challenge materials. The present study investigated the effects of baking on residual milk allergens within baked muffins using recently developed immunoassays for Bos d 5 and Bos d 11[16]. The aim was to compare major allergen levels in uncooked and baked muffin mix that was prepared using two recipes that are commonly used for OFC. IgE reactivity to the uncooked and baked muffin was also compared.

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Methods

Muffin Recipes

Two recipes for baked milk challenge muffin materials were selected for this study: the Mount Sinai recipe, developed at the Jaffe Food Allergy Institute, Icahn School of Medicine at Mount Sinai, New York; and a recipe developed by the Imperial College Healthcare National Health Service (NHS) Trust in the UK[6]. A detailed description of the Mount Sinai muffin recipe has recently been published and this recipe was closely followed in the present study[6]. A comparison of the lists of ingredients, amounts used, baking times and baking temperatures for the Mount Sinai and Imperial College NHS Trust recipes is provided in Table S.1 (supporting information). Ingredients were obtained from a local supermarket in the UK and cooked in a domestic kitchen by one of the researchers involved in the study, to mimic the conditions that would be used by families of patients undergoing OFCs. Muffins were baked at 350°F (180°C) for 30-35 mins (Mount Sinai, n=30) or 20-25 mins (NHS Trust, n=15). All muffins were prepared by the same person in the same kitchen and compared with uncooked muffin mix (n=6).

Extraction of muffin samples

Each baked muffin was divided into 3 sections by hand into a top layer (crust), middle layer and bottom layer (crust) (Figure 1). From each layer, 1 gram of sample was weighed out, finely chopped using clean scissors, and deposited into a 15ml centrifuge tube. Muffin samples were extracted in 10ml of pre-warmed (60°C) phosphate buffered saline, pH 7.4, containing 2% Tween-20 and 1.5M NaCl for 30 minutes in a shaking incubator at 60°C and then centrifuged for 20 minutes at 2500rpm (1250 rcf). The supernatant was pipetted into 1.5ml microcentrifuge tubes and stored at -20°C. Uncooked muffin mix (1g) was extracted using the same protocol.

Quantification and validation of milk allergen measurements

Specific milk allergens were compared using two recently developed two-site monoclonal antibody (mAb) ELISAs for Bos d 5 and for Bos d 11. The Bos d 5 ELISA used

This article is protected by copyright. All rights reserved

mAb NBD5-1 for allergen capture and biotinylated mAb NBD5-2 for detection[18;19]. The capture and biotin detector mAb for the Bos d 11 ELISA were mAb CC11 and VB1C, respectively. The milk allergen specific mAb were developed at the Insitut Nationale des Researches Agricole, Food Allergy Laboratory, Gif sur Yvette, France. Purified nBos d 5 and nBos d 11 were used as assay standards for the ELISA. Protein content was determined by amino acid analysis and purity was validated by mass spectrometry. The lower limit of detection (LLOD) of the ELISA were 0.19ng/ml for Bos d 5 and 7.8ng/ml for Bos d 11. This equates to LLOD of 1.9 ng/g of muffin for Bos d 5 and 78 ng/g for Bos d 11. Both assays were validated in house for linearity, limits of quantification, accuracy and precision (see: https://inbio.com/images/pdfs/ELISA-2.0-Validated-Performance-Data.pdf). The specificity of the Bos d 11 ELISA was established using highly purified natural Bos d 11, which was >99% pure when analysed by mass spectrometry. Parallel binding curves were obtained using purified natural Bos d 11, Bos d 8, baked muffin extract, uncooked muffin extract, and two milk reference materials which were obtained from the National Institute for Standards and Technology (NIST, Gaithersburg, MD), whole milk powder designated NIST SRM 1549a and the MoniQA Association (Neutal, Austria), skim milk powder designated MQA 092014 (Figure 2). Bos d 8 is a composite casein, containing alpha, beta and kappa caseins (Bos d 11). The data confirmed that the Bos d 11 ELISA measured immunoreactive natural allergen in milk. The purity and/or composition of Bos d 8, Bos d 11 and NIST SRM 1549a were compared by mass spectrometry (LC-MS MS on a Thermofisher Q-Exactive instrument) (Table S.2, supporting information).

Variability between allergen levels in uncooked versus baked samples and in different recipes was compared using an unpaired t test. To assess intra-muffin variability (top, middle and bottom of muffin), a one-way ANOVA with Tukey's multiple comparison test was used. Statistical analyses and graphs were prepared using the Graphpad Prism 7 software package.

Binding of IgE antibodies to milk allergens in muffin extracts

This article is protected by copyright. All rights reserved

IgE antibody binding to milk allergens was measured by chimeric ELISA as described previously[20]. Microtiter plates were coated with uncooked or baked muffin mix (0.5µg/well) and incubated with sera from milk-sensitised donors (eight individual sera and one serum pool from 3 milk-sensitised donors). Bound IgE antibodies were detected using peroxidase conjugated goat anti-human IgE (KPL, Gaithersburg, MD) at 1/4000 dilution. Direct binding of IgE antibodies bound to the muffin mix was quantified from a standard curve created using an anti-Der p 2 chimeric IgE antibody bound to Der p 2 coated wells[20].

Inhibition of IgE antibody binding to Bos d 11 by muffin extracts

For the IgE inhibition experiments, microtiter plates were coated with anti-Bos d 11 mAb CC11 (1µg/ml) and incubated with 150ng purified nBos d 11. Sera from milk-sensitized donors were added to each well at a 1/10 dilution and bound IgE antibody was detected using peroxidase conjugated anti-IgE. Binding of IgE antibody to Bos d 11 was inhibited using 100µl of uncooked muffin or baked muffin extract diluted 1/10 – 1/800. Bound IgE antibodies were detected as described above. The protein concentrations of the uncooked and baked muffin extracts were 3.6 mg/ml and 2.7 mg/ml, respectively, by Advanced Protein Assay (Cytoskeleton Inc., Denver, CO). As a control for non-specific inhibition using baked milk extract, inhibition by muffin extract prepared using a dairy free muffin mix (Enjoy Life Baking Mixes, www.amazon.com) was tested. No significant inhibition of IgE binding was observed (<20% inhibition) on testing using 3 milk-allergic sera (data not shown).

Sera from milk-sensitised donors were purchased from commercial serum providers. The sera were obtained from adult donors (age 18-65) and contained high levels of IgE antibodies to milk as determined by ImmunoCAP (range 10-403 kUA/L) (Table S.3, supporting information). Three sera also contained significant levels of IgE to egg and to wheat. The sera used in the direct binding and inhibition experiments were selected based on levels of IgE antibodies to milk and of specific IgE antibodies to Bos d 5 and to Bos d 11 (Tables S.3 and S.4 supporting information).

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Results

Effect of baking on specific allergen levels in muffins

Initially, specific milk allergen levels in uncooked mix and in baked muffin mix prepared using the Mount Sinai recipe were compared. The level of Bos d 5 was reduced from a mean of 680µg/g in uncooked muffin mix to 0.17µg/g in baked muffins, representing more than a 99% decrease after baking (Figure 3.A). Conversely, the level of Bos d 11 was only decreased by 30% from a mean of 4249µg/g in uncooked muffin mix to 2961µg/g in baked muffin (Figure 3.B). This suggested that within baked muffins the level and, therefore, the immunogenicity of the major milk allergen Bos d 11 was mostly unaffected by baking at 350°F (180°C). Indeed, the mean calculated amount of allergen per gram baked muffin was 10.4µg for Bos d 5 and 180,621µg (or 181mg) for Bos d 11 (Table I). During baked milk challenges, patients typically ingest escalating doses ranging from approximately one eighth, or one quarter of a muffin, up to one whole muffin, i.e. ~22.5mg, ~45mg, or ~180mg of immunoreactive Bos d 11, respectively (Table I)[15].

To further assess the effect of baking and temperature, Bos d 11 levels were compared using the top and bottom outer layers and the core (middle) region of baked muffin by sampling 1g from these different sections. Bos d 5 was effectively denatured throughout the top, middle and bottom of the baked muffin (Figure 3.C). The levels of Bos d 11 in the middle of the baked muffin most closely resembled the uncooked mix, whereas the top and bottom outer layers showed significant reductions (~80%) in Bos d 11 (Figure 3.D). This suggested that baking partially denatured Bos d 11 at the periphery and had little effect on Bos d 11 in the remainder of the muffin.

Variability of Bos d 11 levels in baked muffin

Bos d 11 levels were compared in six batches (n=5 per batch) of muffins prepared using the same recipe, ingredients, oven and baker: Bos d 11 varied from 1391-6571µg/g or by up to 4.7-fold (Figure 4.A). Differences between the amount of milk allergen used to challenge patients in the US or in the UK were examined by comparing Bos d 11 levels in muffins prepared using the Mount Sinai or the NHS Trust recipes (Table S.1, supporting

This article is protected by copyright. All rights reserved

information)[6;14]. The results showed significantly higher levels of Bos d 11 in muffins prepared using the Mount Sinai recipe (n=30, mean 2961µg/g) compared with those baked using the NHS Trust recipe (n=15, 1245µg/g, $p<0.0001$) (Figure 4.B). On average, Mount Sinai muffins had 2.4-fold or 58% higher levels of Bos d 11 compared with baked muffins made using the NHS Trust recipe.

Specific IgE reactivity to baked muffin

To corroborate the monoclonal antibody-based ELISA data that baked muffins retained high amounts of immunoreactive allergen, IgE antibody binding to uncooked and baked muffin samples was compared using a pool of sera from three milk-sensitised donors and additional sera from individual milk-sensitised donors (n=8). The results showed that baked muffins consistently bound IgE anti-milk antibodies in direct binding ELISA, retaining ~70% of the IgE reactivity observed in uncooked muffin mix using the serum pool (Figure 5.A). In keeping with the Bos d 11 ELISA results, the highest IgE reactivity was observed in the middle of the muffin compared to the top and bottom (Figure 3). Almost identical IgE binding curves for uncooked muffin and baked muffin were obtained using three individual sera showing little difference in IgE binding after the muffin mix was baked (Figure 5.B). Inhibition ELISA experiments using four individual sera gave similar slopes for both uncooked and baked muffin extracts (Figure 5.C). Baked muffin extract inhibited IgE antibody binding to uncooked muffin mix by 64-96% (Table S.4 supporting information). These results show significant retention of *in vitro* IgE reactivity to Bos d 11 in muffins baked at 350°F (180°C).

Accepted Article

This article is protected by copyright. All rights reserved

Discussion

Although the use of baked milk products for the management of milk allergy has been recommended for 10 years, there have been comparatively few studies on the effect of baking on milk protein allergens. Previous reports using gel electrophoresis and immunoblotting suggested that Bos d 4 (α-lactalbumin) and Bos d 5 (β-lactoglobulin) were heat sensitive, whereas caseins were heat resistant[21]. In keeping with this, casein was resistant to boiling for 60 minutes[6;21]. None of these studies measured the effect of heating on the allergen content of baked milk challenge materials. In the present study, two specific milk allergens (Bos d 5 and Bos d 11) were measured in muffin mixes that are commonly used for OFC. Quantitative data on the concentrations and amount of each allergen in muffin mix were directly compared. The results show that Bos d 5 was readily denatured by baking at 350°F (180°C), whereas Bos d 11 levels remained high in baked muffins, typically retaining ~70% of the immunoreactivity of uncooked muffin mix. The ELISA data was further supported by IgE antibody binding studies which showed that IgE reactivity to baked muffin was retained after baking and that IgE reactivity to uncooked muffin mix was inhibited by baked muffin extract. The IgE data is not consistent with previous reports that foods containing baked milk are less allergenic[6;11;14;15]. While some loss in allergenicity may occur because of denaturation of Bos d 5, the levels of Bos d 11 in uncooked muffin extract were six-fold higher than Bos d 5 and may pose risks for some milk-allergic patients. Baked muffins contained ~181mg Bos d 11. This high level of retained immunogenicity represents a risk for milk allergic patients who are sensitized to Bos d 11 (and possibly other milk caseins).

Sampling of the top, bottom and middle layers of baked muffins revealed, not unexpectedly, that Bos d 11 was more denatured (~80%) at the top and bottom layers where the muffin is more exposed to the direct heat of the oven. Bos d 11 in the middle of the muffin was relatively unaffected. After baking for 35 minutes at 350°F (180°C), the temperatures of the middle (core) of 3 baked muffins (Mount Sinai recipe) were measured at 212.7-214.0°F, (100.4-101.1°C) (data not shown). This lower temperature, for a short time, in a complex matrix, appears to explain in part why Bos d 11 retains its allergenicity, in

This article is protected by copyright. All rights reserved

Accepted Article

addition to the intrinsic heat resistant properties of milk casein proteins. This finding also suggests that a better food challenge material may be one whereby heating is more evenly distributed throughout – for example, a thin waffle or cookie.

These laboratory findings have several implications. First, baked muffins are a reasonably consistent challenge material based on milk allergen levels. The Mount Sinai and NHS Trust recipes varied by 2.4-fold in Bos d 11 levels and are routinely used for successful oral food challenges. Batch to batch variability between the Mount Sinai recipe muffins was within a 4.7-fold range. Greater consistency of baked milk challenge material could be made using commercial food processes and processors that allow more sustained heating throughout the material, yielding a more uniform level of allergen. This may improve consistency when the challenge material is split for dosing regimens that involve increment escalation, either by doubling of the dose every 15-30 minutes i.e. from ⅛ to ¼ to ½ etc; or by increasing the incremental dose using the logarithmic mean i.e. 1, 3, 10, 30, 100, both of which are described in position papers from the European Academy of Allergology and Clinical Immunology[22;23]; or in the 4-dose or 6-dose OFC options recently recommended by the Adverse Reactions to Foods Committee of the AAAAI[14]. Measurement of specific allergens should help to improve the safety of milk and other food products used for OFC. A novel ELISA for Gly m 4 has been used to measure eliciting doses in soy protein challenge matrices and showed a better correlation with patients sensitivity than total soy protein measurements.[24]

Second, the present studies contribute to the growing body of evidence that IgE antibodies to caseins are a risk factor for reactivity to baked milk and increase the likelihood that such patients will fail OFC[6;25]. High total IgE and specific IgE antibodies to casein, high IgE: IgG4 ratios and increased basophil reactivity are all indicators that milk allergic patients will not tolerate baked milk products or regular milk[6;26;27]. Casein-specific IgE of >20kU/L were shown to be predictors of reactivity to baked milk in a cohort of 225 milk-allergic patients and had greater predictive value than specific IgE to cow's milk or to β-lactoglobulin[25]. It was assumed that the risks associated with these high IgE antibody levels were because casein is a major allergen and retained its allergenicity after heating.

This article is protected by copyright. All rights reserved

However, previously no measurements of specific allergen content of challenge materials had been made. Our data provide proof that baked muffins contain high levels of Bos d 11 that react with IgE antibodies and support the assumption made above. Even sera with lower levels of anti-Bos d 11 antibodies (10-40 kUA/L) bound to baked muffin. The quantity of Bos d 11 in baked muffins suggests that patients with >20kU/L IgE to casein should not be challenged with muffin or with similar challenge materials containing baked milk. Our findings indicate that patients who are allergic to Bos d 11 are potentially at greater risk of reacting to baked milk challenge materials. While ~70-75% of milk allergic patients have been reported to tolerate baked milk, a recent systematic review concluded that the evidence that the use of baked milk (or egg) accelerated the resolution of these allergic conditions was weak[27]. The review noted that prior studies were observational and lacked an appropriate control group and cited examples of severe reactions to baked products. A recent long term follow up study also reported that adverse reactions to baked milk were common, with 28% of patients practicing milk avoidance 2-7 years after their initial baked milk challenge[27]. Our data showing high levels of Bos d 11 in baked milk muffins may help to explain these outcomes. The extent to which these adverse reactions are casein-mediated warrants further investigation.

Third, our studies do not explain why a significant number of milk-allergic patients tolerate baked milk while having IgE antibodies to caseins and ingesting baked milk foods with high levels of allergenic Bos d 11. The rationale that these patients have IgE to conformational epitopes that are destroyed by heating seems unlikely based on current understanding of the structural biology of caseins. These belong to a class of 'intrinsically disordered proteins" (IDP) that lack stable three-dimensional structure (and whose structures are unknown)[29;30]. Caseins transport calcium and can assemble as casein micelles which could potentially have conformational or linear epitopes or both[31]. Traditional thinking about conformational and linear epitopes may not apply to caseins or other IDP's. Monoclonal antibody-based immunoassays typically target conformational epitopes, but that may not apply in this case[32;33]. Bos d 11 could readily be measured by ELISA after baking, suggesting that its epitopes were unaffected by baking. Similarly, IgE reactivity remained

This article is protected by copyright. All rights reserved

after baking suggesting that the IgE epitopes recognised by these patients were retained. Baking is reported to have no significant impact on dose distribution curves for milk protein reaction thresholds[34]. It has been proposed that patients who tolerate baked milk have a phenotype that is less likely to result in persistence of milk allergy[35]. However, the attributes of this particular phenotype are unclear. A more nuanced hypothesis for baked milk tolerance is that it represents a balance between the amount of Bos d 11 (and other caseins) in the food; the quantity and affinity of casein-specific IgE; the sensitivity to mast cell and basophil histamine release; and factors affecting absorption of milk proteins in the gut associated lymphoid tissue.

The limitations of the present study are that the Bos d 11 ELISA used in this study may not react to other caseins which only share 9-25% sequence homology. The Bos d 11 ELISA may underestimate the total level of casein allergens. A paucity of serum samples from milk-sensitized donors limited the scope of the IgE binding studies. Three sera also contained IgE antibodies to egg and to wheat by ImmunoCAP. However, other sera contained high levels of IgE to milk (>100 kUA/L) with negligible binding to egg or wheat, suggesting that IgE binding to baked muffin was unrelated to egg or wheat sIgE. The sera were all from milk-sensitized adults which may bias the study population towards individuals with persistent milk allergy. With regard to symptoms, the sera were obtained from donors with self-reported sensitivity to milk, which was verified by the sIgE measurements. The study would have been strengthened by using sera from patients with physician-diagnosed, clinical milk allergy with a full diagnostic work up, including OFC. This would also allow for adverse reactions to milk to be studied in the context of sIgE levels to Bos d 11 and the amount of Bos d 11 in milk challenges. Our study lays the groundwork for future studies of patients with different clinical manifestations of milk allergy. Overall, these limitations did not affect the underlying conclusion that baked muffins used for OFC contain large quantities of the major milk allergen, Bos d 11.

In conclusion, the levels of the major milk allergen, Bos d 11 (β-casein), remain high within baked muffins used for OFC. Most of the sIgE reactivity to Bos d 11 remained after baking. The data suggests that high levels of Bos d 11 in baked milk muffins and other

Accepted Article

This article is protected by copyright. All rights reserved

baked milk products could contribute to adverse reactions upon OFC and on introduction of baked milk products into the diet. Molecular diagnostics could be used to screen patients for specific IgE antibodies to caseins and potentially to exclude patients with high IgE levels from food challenges based on a risk assessment. Patient safety could be further improved using molecular analysis of food allergens to determine the dose and consistency of food challenge materials, coupled with molecular diagnostics, as part of an integrated approach to risk assessment of OFC.

Conflict of Interest Statement

JH, MO and AC are employees of Indoor Biotechnologies Ltd (Cardiff, UK). CT and SW are employees of Indoor Biotechnologies Inc (Charlottesville, VA, USA). M. Chapman is an owner and an employee of both companies.

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

References

(1) Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA et al. Guidelines for the diagnosis and management of food allergy in the United States: Report of the NIAID-sponsored expert panel. *J Allergy Clin Immunol* 2010; **126**(6):S1-58.

(2) Grimshaw KEC, Bryant T, Oliver EM, Martin J, Maskell J, Kemp T et al. Incidence and risk factors for food hypersensitivity in UK infants: results from a birth cohort study. *Clin Transl Allergy* 2016; **6**.

(3) Peters RL, Koplin JJ, Gurrin LC, Dharmage SC, Wake M, Ponsonby AL et al. The prevalence of food allergy and other allergic diseases in early childhood in a population-based study: HealthNuts age 4-year follow-up. *J Allergy Clin Immunol* 2017; **140**(1):145-53.

(4) McGowan EC, Peng RD, Salo PM, Zeldin DC, Keet CA. Changes in Food-Specific IgE Over Time in the National Health and Nutrition Examination Survey (NHANES). *J Allergy Clin Immunol Pract* 2016; **4**(4):713-20.

(5) Kim JS, Nowak-Wegrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked-milk accelerates resolution of cow's milk allergy in children. *J Allergy Clin Immunol* 2011; **128**(1):125-31.

(6) Leonard SA, Caubet JC, Kim JS, Groetch M, Nowak-Wegrzyn A. Baked milk-and egg-containing diet in the management of milk and egg allergy. *J Allergy Clin Immunol Pract* 2015; **3**(1):13-23.

(7) Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S et al. *EAACI Molecular Allergology User's Guide. Pediatr Allergy Immunol* 2016; **27**(23):1-250.

(8) Nowak-Wegrzyn A, Muraro A. Cows milk allergy. Matricardi PMea, editor. *EAACI Molecular Allergology User's Guide. Pediatr Allergy Immunol* 2016; **27**(23):1-250.

This article is protected by copyright. All rights reserved

Accepted Article

(9) EFSA Panel on Dietetic Products, Nutrition and Allergies. Scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. *EFSA Journal* 2014; **12**(11):3894:1-286.

(10) Robbins KA, Wood RA, Keet CA. Milk allergy is associated with decreased growth in U.S. children. *J Allergy Clin Immunol* 2014; **134**(6):1466-8.

(11) Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N et al. Tolerance to extensively heated milk in children with cow's milk allergy. *J Allergy Clin Immunol* 2008; **122**(2):342-7, 347.

(12) Vila L, Beyer K, Jarvinen KM, Chatchatee P, Bardina L, Sampson HA. Role of conformational and linear epitopes in the achievement of tolerance in cow's milk allergy. *Clin Exp Allergy* 2001; **31**(10):1599-606.

(13) Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2002; **110**(2):293-7.

(14) Bird JA, Leonard S, Groetch M, Assa'ad A, Cianferoni A, Clark A et al. Conducting an Oral Food Challenge: An Update to the 2009 Adverse Reactions to Foods Committee Work Group Report. *J Allergy Clin Immunol Pract* 2020; **8**(1):75-90.

(15) Bartnikas LM, Sheehan WJ, Hoffman EB, Permaul P, Dioun AF, Friedlander J et al. Predicting food challenge outcomes for baked milk: role of specific IgE and skin prick testing. *Ann Allergy Asthma Immunol* 2012; **109**(5):309-13.

(16) Roberts G. Food challenges, the risks and benefits. *Clinical and Experimental Allergy* 2017; **47**(9):1106-7.

(17) Niggemann B, Yurek S, Beyer K. Severe anaphylaxis requiring intensive care during oral food challenge-It is not always peanuts. *Pediatr Allergy Immunol* 2017; **28**(2):201-3.

This article is protected by copyright. All rights reserved

(18) Negroni L, Bernard H, Clement G, Chatel JM, Brune P, Frobert Y et al. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. *J Immunol Methods* 1998; **220**(1-2):25-37.

(19) Clement G, Boquet D, Frobert Y, Bernard H, Negroni L, Chatel JM et al. Epitopic characterization of native bovine beta-lactoglobulin. *J Immunol Methods* 2002; **266**(1-2):67-78.

(20) Trombone AP, Tobias KR, Ferriani VP, Schuurman J, Aalberse RC, Smith AM et al. Use of a chimeric ELISA to investigate immunoglobulin E antibody responses to Der p 1 and Der p 2 in mite-allergic patients with asthma, wheezing and/or rhinitis. *Clin Exp Allergy* 2002; **32**(9):1323-8.

(21) Bloom KA, Huang FR, Bencharitiwong R, Bardina L, Ross A, Sampson HA et al. Effect of heat treatment on milk and egg proteins allergenicity. *Pediatr Allergy Immunol* 2014; **25**(8):740-6.

(22) Bindslev-Jensen C, Ballmer-Weber BK, Bengtsson U, Blanco C, Ebner Cet al. Standardization of food challenges in patients with immediate reactions to foods - position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 2004; **59**(7):690-7.

(23) Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K et al. EAACI food allergy and anaphylaxis guidelines: diagnosis and management of food allergy. *Allergy* 2014; **69**(8):1008-25.

(24) Holzhauser T, Franke A, Treudler R, Schmiedeknecht A et al. The BASALIT multicenter trial: Gly m 4 quantification for consistency control of challenge meal batches and toward Gly m 4 threshold data. Mol. Nutr. Food Res. 61, 3, 2017, 1600527

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

(25) Caubet JC, Nowak-Wegrzyn A, Moshier E, Godbold J, Julie W, Sampson HA. Utility of casein-specific IgE levels in predicting reactivity to baked milk. *J Allergy Clin Immunol* 2013; **131**(1):222-4.

(26) Ford LS, Bloom KA, Nowak-Wegrzyn AH, Shreffler WG, Masilamani M, Sampson HA. Basophil Reactivity, Wheal Size and Immunoglobulin Levels Distinguish Degree of Cows Milk Tolerance. *J Allergy Clin Immunol* 2013; **131**(1):180-6.

(27) Dunlop JH, Keet CA, Mudd, Wood RA. Long-term follow-up after baked milk introduction. *J Allergy Clin Immunol Pract.* 2018; **6**(5):1699-704.

(28) Lambert R, Grimshaw KEC, Ellis B, Jaitly J, Roberts G. Evidence that eating baked egg or milk influences egg or milk allergy resolution: a systematic review. *Clin Exp Allergy* 2017; **47**(6):829-37.

(29) Perticaroli S, Nickels JD, Ehlers G, Mamontov E, Sokolov AP. Dynamics and rigidity in an intrinsically disordered protein, beta-casein. *J Phys Chem B* 2014; **118**(26):7317-26.

(30) Mirdha L, Chakraborty H. Characterization of structural conformers of kappa-casein utilizing fluorescence spectroscopy. *Int J Biol Macromol* 2019; **131**: 89-96.

(31) Redwan EM, Xue B, Almehdar HA, Uversky VN. Disorder in milk proteins: caseins, intrinsically disordered colloids. *Curr Protein Pept Sci* 2015; **16**(3):228-42.

(32) Pomes A, Chruszcz M, Gustchina A, Minor W, Mueller GA, Pedersen LC et al. 100 Years later: Celebrating the contributions of x-ray crystallography to allergy and clinical immunology. *J Allergy Clin Immunol* 2015; **136**(1):29-37.

(33) Pomes A, Chruszcz M, Gustchina A, Wlodawer A. Interfaces between allergen structure and diagnosis: know your epitopes. *Curr Allergy Asthma Rep* 2015; **15**(8):506.

This article is protected by copyright. All rights reserved

(34) Remington BC, Westerhout J, Campbell DE, Turner PJ. Minimal impact of extensive heating of hen's egg and cow's milk in a food matrix on threshold dose-distribution curves. *Allergy* 2017; **72**(11):1816-9.

(35) Dang TD, Peters RL, Allen KJ. Debates in allergy medicine: baked egg and milk do not accelerate tolerance to egg and milk. *World Allergy Organ J* 2016; **9**.

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Legends to Figures

Figure 1. Full (A) and cut away (B) images of a typical muffin prepared and baked using the Mount Sinai recipe. Dashed lines also show the areas considered to be the top, middle and bottom of the muffin.

Figure 2. Comparison of dilution curves using purified Bos d 8 (●), purified Bos d 11 (●), uncooked (■) or baked (■) muffin extract, and NIST (▲) and MoniQA (▲) milk reference materials.

Figure 3. Effect of baking on Bos d 5 (A) and Bos d 11 (B) levels in muffin extracts. Values for uncooked muffin mix were compared with those of muffins baked at 350°F (180°C) for 30-35 mins (Mount Sinai recipe). Mean and SD; uncooked n=6; baked n=30. ****p<0.0001, NS=not significant, using unpaired t test. Bos d 5 (C) and Bos d 11 (D) levels in uncooked muffin mix and baked muffins sampled from the top, middle and bottom of the muffin were compared. Mean and SD; uncooked n=6; baked n=30; p<0.0001 using one-way ANOVA, ****p<0.0001 and NS = not significant for uncooked muffin mix versus top, middle and bottom of baked muffin using Tukey's comparison test.

Figure 4. A. Batch to batch comparison of Bos d 11 levels in individual baked muffins (6 different batches, 5 muffins per batch. B. Comparison of Bos d 11 levels in muffins prepared using the Mount Sinai (n=30) and NHS Trust (n=15) muffin recipes. Individual muffins and geometric mean, p=0.0001 using unpaired t test.

Figure 5. A. IgE reactivity to uncooked and baked muffins was compared by chimeric ELISA using a serum pool from three milk allergic patients containing IgE antibody to Bos d 5 and Bos d 11. B. Comparison of direct IgE binding ELISA dilution curves of three sera (#'s 19230 (●,●), 21527 (■,■), and 24775 (▲,▲) to uncooked (solid blue symbols/lines) or baked muffin extract (dashed red symbols/lines). C. Dose dependent inhibition of IgE antibody binding to Bos d 11 by uncooked muffin mix (solid blue symbols/lines) or by baked muffin (dashed red symbols/line) using four sera (#'s 18023-B (■,■), 19230-J (▲,▲), 21527-L (●,●) and Ab-E16322 (♦,♦).

This article is protected by copyright. All rights reserved

Accepted Article

This article is protected by copyright. All rights reserved

Table I

Specific Milk Allergen Concentrations in Baked Muffin

	Mean Allergen Level	
	Bos d 5	**Bos d 11**
Allergen concentration (µg/g)†	0.17	2,961
Allergen content in 1 baked muffin (µg)‡	10.37	180,621
Allergen content in ¼ of baked muffin (µg)	2.59	45,155
Allergen content in ⅛ of baked muffin (µg)	1.30	22,578

†Mean allergen concentration based on muffins prepared using the Mount Sinai recipe (n=30).

‡Based on a mean muffin weight of 61g (n=6).

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Figure 1

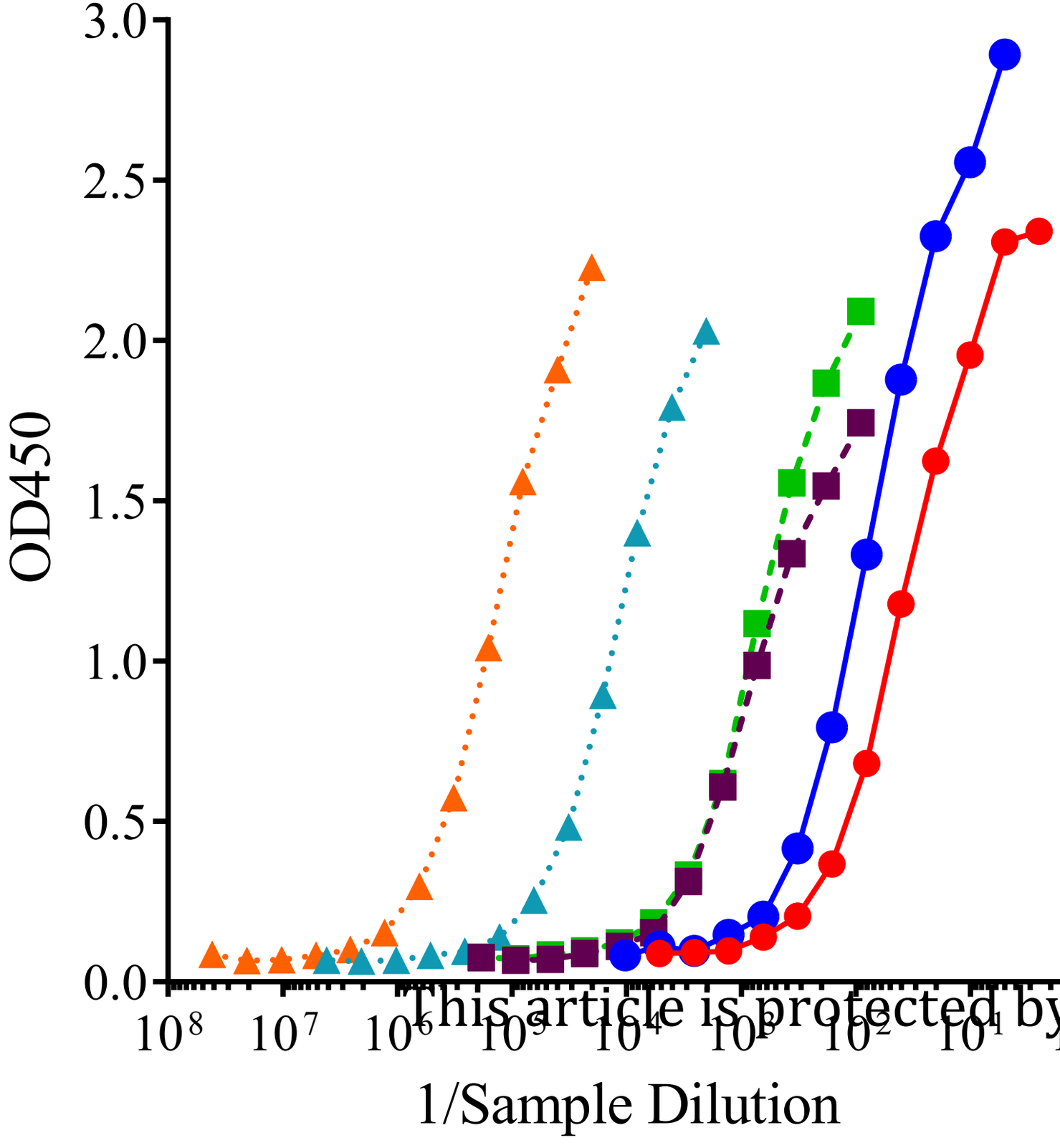
A. B.



cea_13774_f1.tif

This article is protected by copyright. All rights reserved

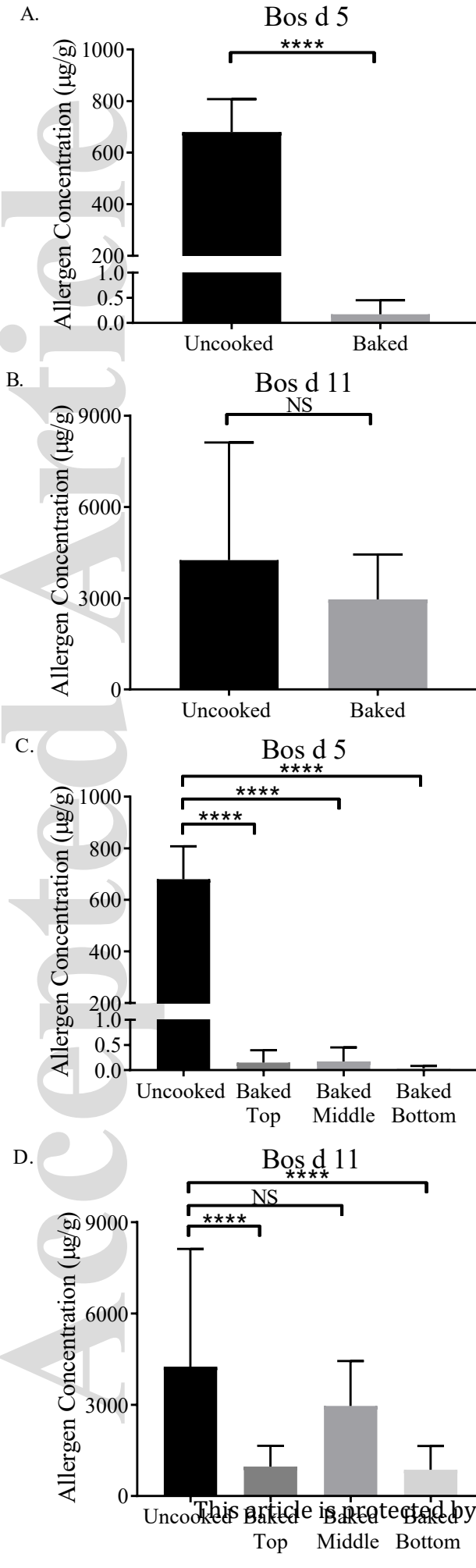
Figure 2 cea_13774_f2.pdf



This article is protected by copyrig

Accepted Article

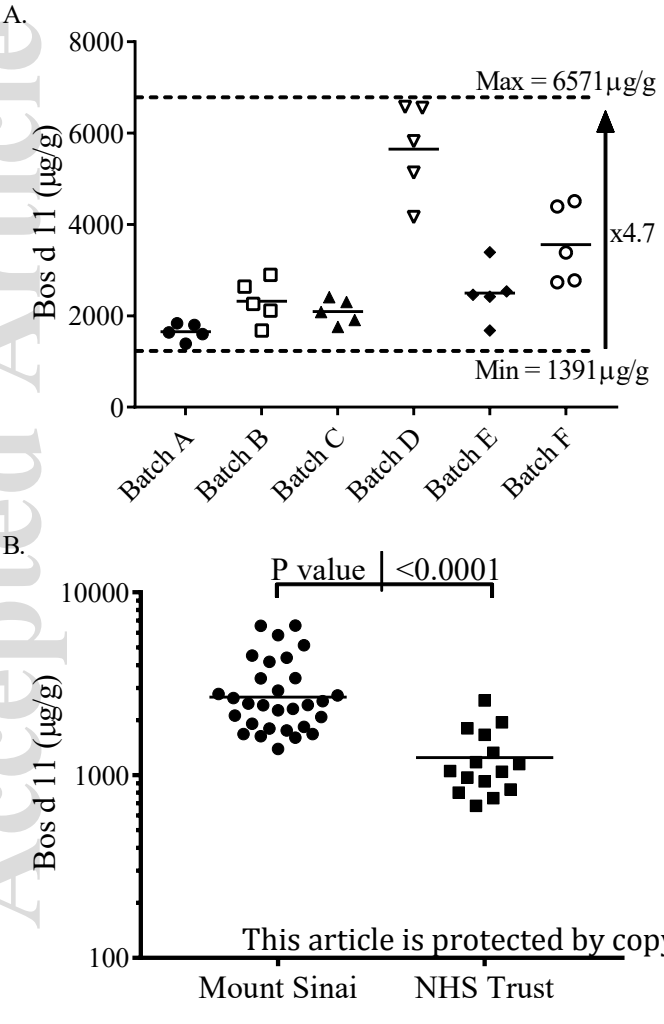
Figure 3 cea_13774_f3.pdf



Accepted Article

This article is protected by copyrig

Figure 4 cea_13774_f4.pdf

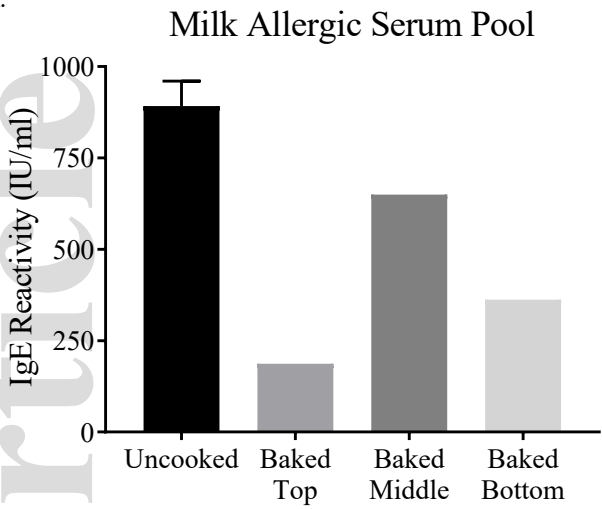


Accepted Article

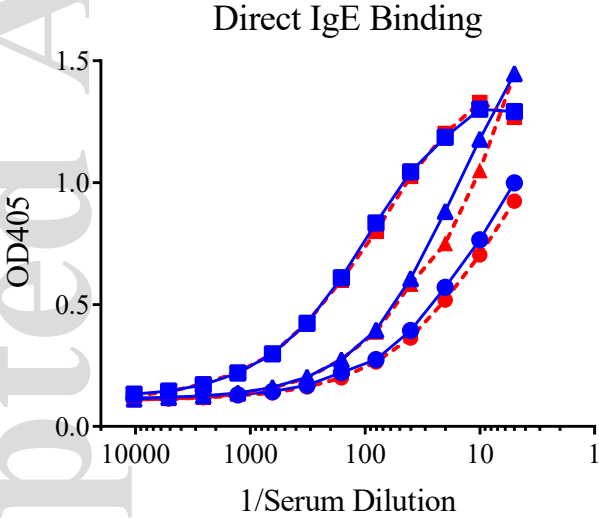
This article is protected by copyright

Figure 5 cea_13774_f5.pdf

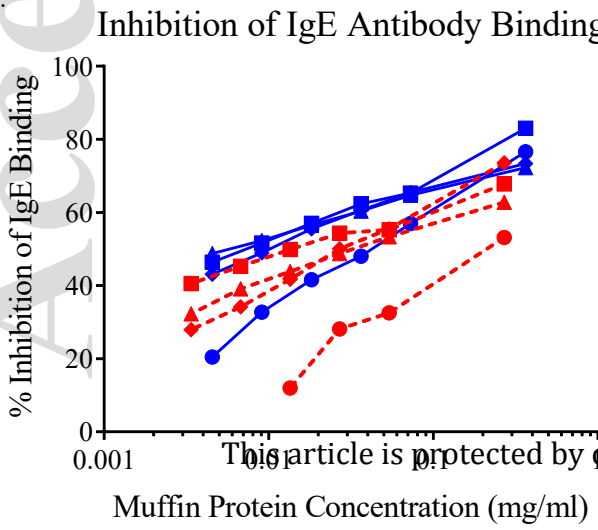
A.



B.



C.



Accepted Article

This article is protected by copyrigh

Supporting Information

Bos d 11 in baked milk poses a risk for adverse reactions in milk allergic patients.

James P Hindley PhD[1], Maria Oliver PhD[1], Cathy Thorpe BS[2], Angharad Cullinane BSc[1], Sabina Wuenschmann PhD[2], Martin D Chapman PhD[2]

[1] Indoor Biotechnologies Ltd, Cardiff, UK

[2] Indoor Biotechnologies Inc, Charlottesville, VA, USA

Corresponding Author:

James P Hindley PhD

Indoor Biotechnologies Ltd,

Vision Court, Caxton Place

Cardiff, CF23 8HA

United Kingdom

hindleyjp@indoorbiotech.co.uk

+44 (0) 2921 674 640

Table S.1

Comparison of baked milk muffin recipes developed at Mount Sinai or the Imperial College NHS Trust.

Recipe Comparison Table	
Mount Sinai	Imperial College NHS Trust
Yields 6 muffins (1.3g cow's milk per muffin)	Yields 10 muffins
Ingredients	Ingredients
1 cup cow's milk †[240ml]	250ml cow's milk
2 tablespoons canola oil	125g butter at room temperature
1 egg or 1-1/2 tsp egg replacer (e.g. Ener-G brand) †[1 medium sized egg used]	2 eggs or egg substitute †[2 medium sized eggs used]
1 tsp vanilla extract	1 teaspoon vanilla essence optional †[1 teaspoon vanilla essence used]
1/2 cup sugar †[100g caster sugar used]	160g caster sugar
1 1/4 cups flour †[150g plain flour used]	200g sifted self-raising flour (or gluten free flour with 1 teaspoon baking powder) †[200g sifted self-raising flour used]
1/4 tsp salt	
2 tsp baking powder	
Directions	Directions
1. Preheat oven to 350 degrees Fahrenheit †[180°C]	1. Pre-heat conventional oven to 180 degrees (165 fan) †[180°C]
2. Line a muffin pan with 6 muffin liners.	2. Add softened butter and sugar and mix on low speed until creamy

3. Mix the liquid ingredients: milk, canola oil, vanilla extract, and egg. Set aside.	3. Add the two eggs and mix on low speed until combined
4. In a separate bowl, mix the dry ingredients: flour, sugar, salt, and baking powder. Set aside.	4. Add alternative equal amounts of sifted flour and milk whilst mixing on low speed until all flour and milk has been added
5. Add the dry ingredients to the liquid ingredients. Stir until combined. Some small lumps may remain.	5. Combine ingredients on medium speed until smooth
6. Divide the batter into 6 prepared muffin liners.	6. Add optional 1 teaspoon vanilla essence
7. Bake for 30-35 minutes, or until golden brown and firm to the touch.	7. Place muffin cups into the tray and distribute batter evenly into each of 10 muffin cups
ALTERNATIVE: standard cake mix with 1 cup of cow's milk, where the total challenge dose contains ~1.3 g cow's milk protein	8. Bake for 20-25 minutes or until slightly golden brown and just firm to touch (or test with skewer)
Dosing: 1/8, 1/8, 1/4, 1/2 every 15 minutes.	

Footnotes:

1. Milk challenge recipes from the Jaffe Food Allergy Institute, Icahn School of Medicine at Mount Sinai,New York, USA and from the Imperial College Healthcare NHS Trust (London, UK) were compared.
2. †[in brackets] = Actual measurements, temperatures and ingredients used.
3. Cup to metric value conversion http://www.jsward.com/cooking/conversion.shtml

Table S.2

Relative abundance of specific milk allergens in purified Bos d 8 and Bos d 11 and in NIST milk reference material determined by mass spectrometry.

	Relative Allergen Abundance (%)		
	nBos d 8	**nBos d 11**	**NIST SRM 1549a**
Bos d 4	0.15	--	4.22
Bos d 5	0.71	--	23.49
Bos d 6	0.08	--	0.62
Bos d 9	77.32	0.06	39.51
Bos d 10	5.07	--	13.43
Bos d 11	11.33	99.5	10.94
Bos d 12	5.23	0.35	3.22

Table S.3

Specific IgE Profiles of Sera from Milk Sensitized Donors

S.3.A Serum Pool (n=3)

Serum ID	Milk CAP (kUA/L)	Bos d 5 (kUA/L)	Bos d 8 (kUA/L)	Bos d 11 (IU/ml)
15046	81	3.2	>100	1034
17488	86	42	>100	238
20124	32	10.9	28	107

S.3.B Individual Sera

Serum ID	Age, Gender	Milk CAP (kUA/L)	Bos d 5 (kUA/L)	Bos d 8 (kUA/L)	Bos d 11 (IU/ml)	Egg CAP (kUA/L)	Wheat CAP (kUA/L)
18023-B	29, M	>100	37	75	101	0.1	0.2
19230-J	31, M	42	1.7	48	35	<0.1	<0.1
21527-L	26, M	403	50	473	61	0.7	0.8
24775	65, F	88.4	1.0	430	224	34	38
24777	18, M	10.4	2.7	14	5	26	3.6
Ab-E16322	27, M	65.6	12	250	340	0.8	0.3
Ab-E16631	63, F	88.7	3.0	260	375	31	40
23823	27, M	23.4	4.2	12	6	<0.1	<0.1

Specific IgE to milk was determined by ImmunoCAP (data provided by Plasmalab International, Everett, WA). Specific IgE to Bos d 5, Bos d 8, egg and wheat was determined by ImmunoCAP at Indoor Biotechnologies (Charlottesville, VA). IgE to Bos d 11 was determined by chimeric ELISA.

Table S.4

Inhibition of IgE antibody binding to Bos d 11 by uncooked or baked muffin in ELISA

Serum ID	Uncooked Muffin (%)	Baked Muffin (%)	% retained after baking
18023-B	74	61	82
19230-J	68	57	83
21527-L	66	42	64
24775	77	68	88
24777	53	51	96
Ab-E16322	69	64	93
Ab-E16631	83	75	90
23823	48	34	71

Inhibition of IgE antibody binding was assessed as described in materials and methods. Percent inhibition by baked muffin extract compared with uncooked muffin extract was calculated for each sample.