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Study Protocol - Impact of Lactobacillus rhamnosus GG on Allergic Reactions and Gut Microbiota Composition in Children with Cow's Milk Allergy: A Single-Center, Randomized, Double-Blinded, Placebo-Controlled, Phase II.

MILK ALLERGY Trial

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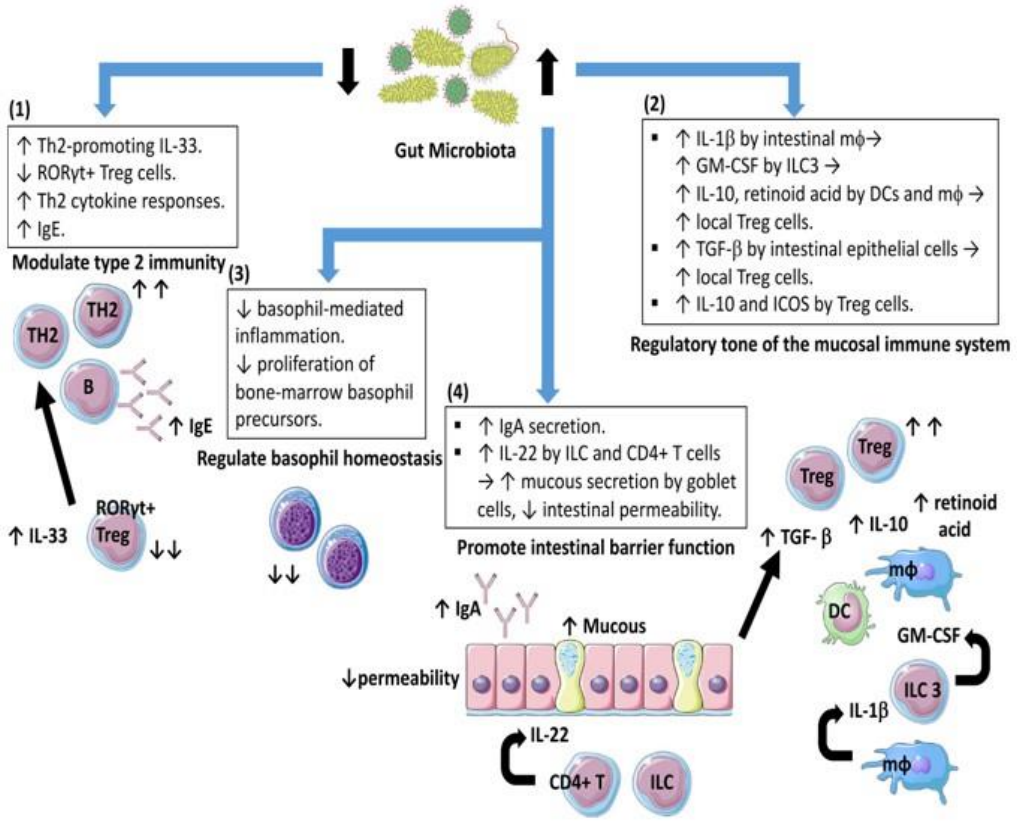
APPENDICES

Appendix A.1.
Mechanisms by which Gut Microbiota Modification Determines Tolerance vs. Susceptibility



⇩ ***(1)****Gut microbiota modulate adaptive immunity.*

⇧ ***(2)****Gut microbiota maintain the regulatory tone of the mucosal immune system.*

⇧ ***(3)****Gut microbiota regulate basophil homeostasis (Ohnmacht & Voehringer, 2009).*

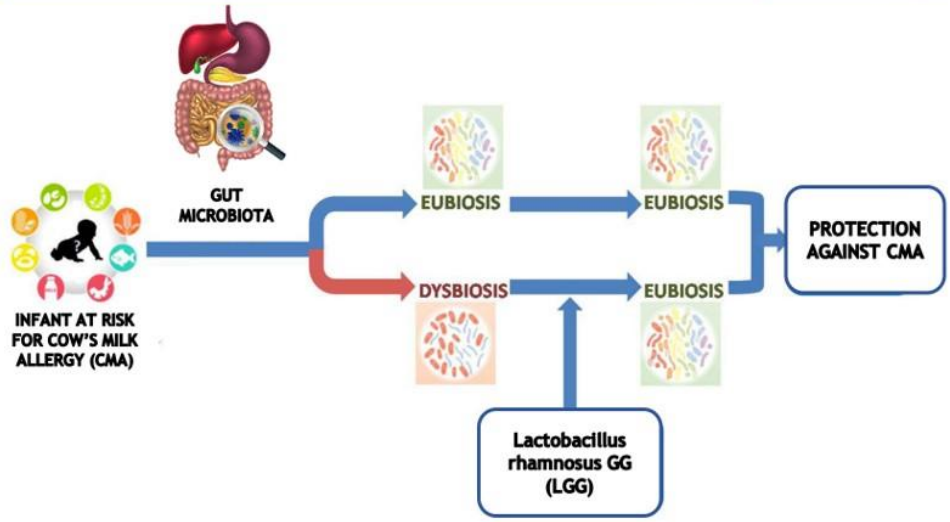
⇧ ***(4)****Gut microbiota promotes intestinal barrier function.*

[TH2] lymphocytes T Helper Type 2 **[IL-33]** cytokine IL-33 **[Treg]** regulatory T cells **[RORγt+]** nuclear hormone receptor RORγt **[IgA]** Immunoglobulin A **[IL-22]** cytokine IL-22 **[CD4+T]** T helper cells **[ILC]** innate lymphoid cells **[DC]** dendritic cells **[ICOS]** Inducible T-cell COStimulator **[TGF-β]** Transforming growth factor beta **[IL-10]** cytokine IL-10 **[mφ]** intestinal macrophages **[GM-CSF]** Granulocyte-Macrophage Colony-Stimulating Factor **[ILC3]** type 3 innate lymphoid cells **[IL-1β]** Interleukin 1 beta.

Adapted from (Ho & Bunyavanich, 2018).

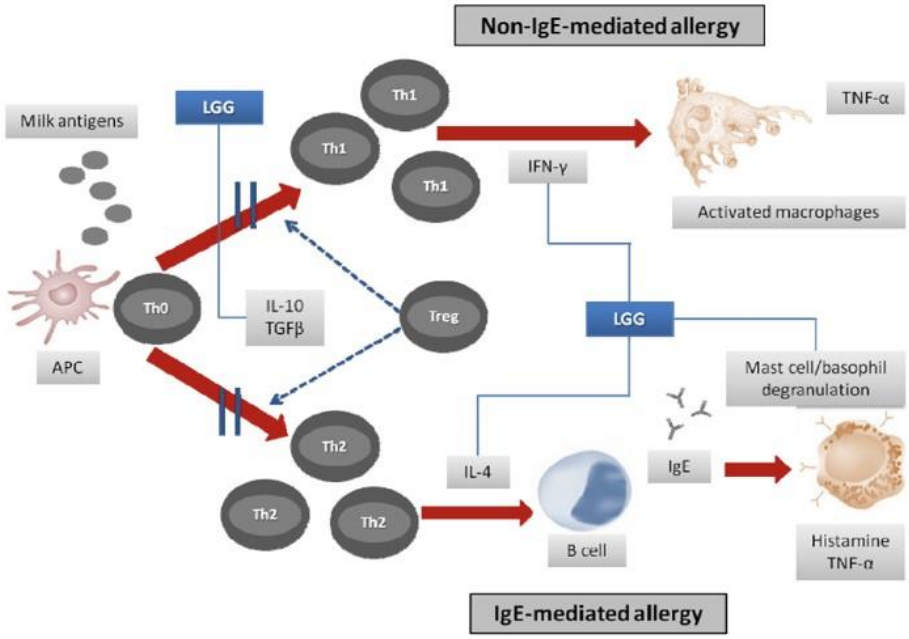
Appendix A.2.
Improvement of CMA: Modification of Gut Microbiota with LGG therapy

Adapted from (Canani et al., 2019)



Appendix A.3.
LGG Acts on Different Mechanisms of CMA

Adapted from (Canani et al., 2012)



[LGG] Lactobacillus rhamnosus GG **[CMA]** Cow's Milk Allergy **[APC]** antigen-presenting cell **[Th0]** naive T cell **[Th1]** lymphocytes T Helper Type 1 **[Th2]** lymphocytes T Helper Type 2 **[Treg]** regulatory T cells **[IFN-γ]** Interferon gamma **[TGF-β]** Transforming growth factor beta **[IL-10]** cytokine IL-10 **[IL-4]** cytokine IL-4 **[TNF-α]** Tumor necrosis factor α.

Appendix B.1

CONSENT FORM

MILK ALLERGY TRIAL

This is a research study for children who are allergic to cow's milk. The purpose of this trial is to see if the use of oral supplements called probiotics can improve the gut bacterial and immunological barrier on your child. With the intention that when your child ingests cow's milk in the future, he/she will not develop any symptoms or develop milder symptoms that permits him/her to eases his/her life and yours as parents.

We will treat them with a probiotic strain called Lactobacillus rhamnosus GG for 12 months, and we will look for clinical manifestations after oral intake of cow's milk and changes in the gut environment after the intervention.

The cow's milk exposure will be done by a standardized and validated method to diagnose food allergies called Oral Food Challenge (OFC), and it can also help determine if the diagnosed food allergy (FA) has resolved or improved after the intervention. This OFC procedure involves consuming gradual amounts of cow's milk under medical supervision. The possible risks and discomforts could include itchy rash, hives, nausea, vomiting, abdominal discomfort, diarrhea, cough, stuffy, runny nose, sneezing, facial swelling, wheezing, and shortness of breath. Major risks could include breathing difficulties and anaphylaxis. The physicians taking care of this process are committed to rigorous safety standards, including graded exposures and careful observation. Medical staff will be fully equipped to treat any symptoms that may occur, including severe ones. The challenge will be stopped if severe reactions or major risks occur. Treatment for reactions may involve administering antihistamines, corticosteroids, and/or epinephrine, observation for up to several hours, visit to an emergency department, or admission to the hospital.

This research study is expected to take approximately 15 months with one visit for screening, including an OFC and feces collection, monthly follow up phone calls, a visit for an OFC and feces collection after 12 months of treatment, and a visit for feces collection three months after finalizing of the intervention.

We cannot and do not guarantee or promise that you or your child will receive any benefits from this study. The knowledge obtained from this clinical study helps in the advancement and understanding of FA and supports the development of new approaches for its treatment or prevention. Also, note that this is a placebo-controlled study; half of the participants will receive a sham treatment. Allocation to the active treatment group or placebo group will be done at random. This is necessary to differentiate and ascertain the effect of the active treatment. The study will also be double-blinded; this means that neither you nor your child nor the research team will know which treatment your child is receiving.

Your child was selected as a possible participant in this study because he/she has a proven IgE-mediated cow's milk allergy. If you decide to terminate your participation in this study, you should notify the research team.

The participation of your child in this study is entirely voluntary. If you decide not to participate, it will not negatively affect his/her or his/her medical care. You can decide that your child participates now but withdraw your consent later and stop participating in the study without any loss of benefits or medical care to which he/she is entitled.

The purpose, risks, benefits, and alternatives of the MILK ALLERGY TRIAL have been explained to my satisfaction. I understand that there is always a possibility of a reaction to the cow's milk intake. I also understand that, as with every procedure, there is a possibility of unexpected complications over my child.

I give my consent for my child to undergo the MILK ALLERGY TRIAL.

I authorize the doctors to treat my child if allergic reactions occur. _____

Patient's Name

_____

Date of Birth

_____

Responsible Party/Guarantor Name

_____

Relationship to Patient

_____

Patient/Responsible Party/Guarantor Signature

_____

Healthcare Provider's Signature

_____

Institution

Date: _____City: _____

Appendix B.2

ASSENT FORM

MILK ALLERGY TRIAL

This is a research study for children who are allergic to cow's milk. The purpose of this trial is to see if the use of oral supplements called probiotics can improve the gut bacterial and immunological barrier in you. With the intention that when you ingest cow's milk in the future, you will not develop any symptoms or develop milder symptoms, permitting you to ease your life and of your parents.

We will treat you with a probiotic strain called Lactobacillus rhamnosus GG for 12 months, and we will look for clinical manifestations after oral intake of cow's milk and changes in the gut environment after the intervention.

The cow's milk exposure will be done by a standardized and validated method to diagnose food allergies called Oral Food Challenge (OFC), and it can also help determine if the diagnosed food allergy (FA) has resolved or improved after the intervention. This OFC procedure involves consuming gradual amounts of cow's milk under medical supervision. The possible risks and discomforts could include itchy rash, hives, nausea, vomiting, abdominal discomfort, diarrhea, cough, stuffy, runny nose, sneezing, facial swelling, wheezing, and shortness of breath. Major risks could include breathing difficulties and anaphylaxis. The physicians taking care of this process are committed to rigorous safety standards, including graded exposures and careful observation. Medical staff will be fully equipped to treat any symptoms that may occur, including severe ones. The challenge will be stopped if severe reactions or major risks occur. Treatment for reactions may involve administering antihistamines, corticosteroids, and/or epinephrine, observation for up to several hours, visit to an emergency department, or admission to the hospital.

This research study is expected to take approximately 15 months with one visit for screening, including an OFC and feces collection, monthly follow up phone calls, a visit for an OFC and feces collection after 12 months of treatment, and a visit for feces collection three months after finalizing of the intervention.

We cannot and do not guarantee or promise that you will receive any benefits from this study. The knowledge obtained from this clinical study helps in the advancement and understanding of FA and supports the development of new approaches for its treatment or prevention. Also, note that this is a placebo-controlled study; half of the participants will receive a sham treatment. Allocation to the active treatment group or placebo group will be done at random, and this is necessary to differentiate and ascertain the effect of the active treatment. The study will also be double-blinded; this means that neither you nor the research team will know which treatment you are receiving.

You were selected as a possible participant in this study because you have a proven IgE-mediated cow's milk allergy. If you decide to terminate your participation in this study, you and your parents should notify the research team.

Your participation in this study is entirely voluntary. If you decide not to participate, it will not negatively affect you or your medical care. You can decide to participate now but withdraw your consent later and stop participating in the study without any loss of benefits or medical care to which you are entitled.

The purpose, risks, benefits, and alternatives of the MILK ALLERGY TRIAL have been explained to my satisfaction. I understand that there is always a possibility of a reaction to the cow's milk intake. I also understand that, as with every procedure, there is a possibility of unexpected complications.

I give my assent to undergo the MILK ALLERGY TRIAL.

I authorize the doctors to treat me if allergic reactions occur. _____

Patient's Name

_____

Patient's Signature

_____

Date of Birth

_____

Responsible Party/Guarantor Name

_____

Relationship to Patient

_____

Patient/Responsible Party/Guarantor Signature

_____

Healthcare Provider's Signature

_____

Institution

Date: _____City: _____

Appendix C

MEDICAL HISTORY FORMAT

Center of origin		Date
Name		Contact
Parents		Age
Sex		
Race/Ethnicity:	( )American Indian or Alaska Native ( )White/Caucasian ( )Hispanic/Latino ( )Native Hawaiian or Other Pacific Islander	( ) Asian ( ) Black ( ) Other: _____
Length of pregnancy:		
Birth weight:		
Type of delivery:	( ) Vaginal	
	( ) Planned C Section	
	( ) Emergency C section	
Problems with the pregnancy?	Yes ( ) No ( )	Describe:
Were there problems during the birth?	Yes ( ) No ( )	Describe:
Was your child breast fed?	Yes ( ) No ( )	

Was your child formula fed?	Yes ( ) No ( )	Describe:
Are shots (immunizations) up-to-date?	Yes ( ) No ( )	

Date of Diagnostic of Milk Allergy:

Result of IgE dosage:

Type of Reaction

Upper Respiratory	Yes ( ) No ( )	Describe:
Lower Respiratory	Yes ( ) No ( )	Describe:
Cutaneous	Yes ( ) No ( )	Describe:
Gastrointestinal	Yes ( ) No ( )	Describe:
Ocular	Yes ( ) No ( )	Describe:
Cardiovascular	Yes ( ) No ( )	Describe:
CNS	Yes ( ) No ( )	Describe:

Other diseases:

OTHER ALLERGIC PROBLEMS:

Medications in use:

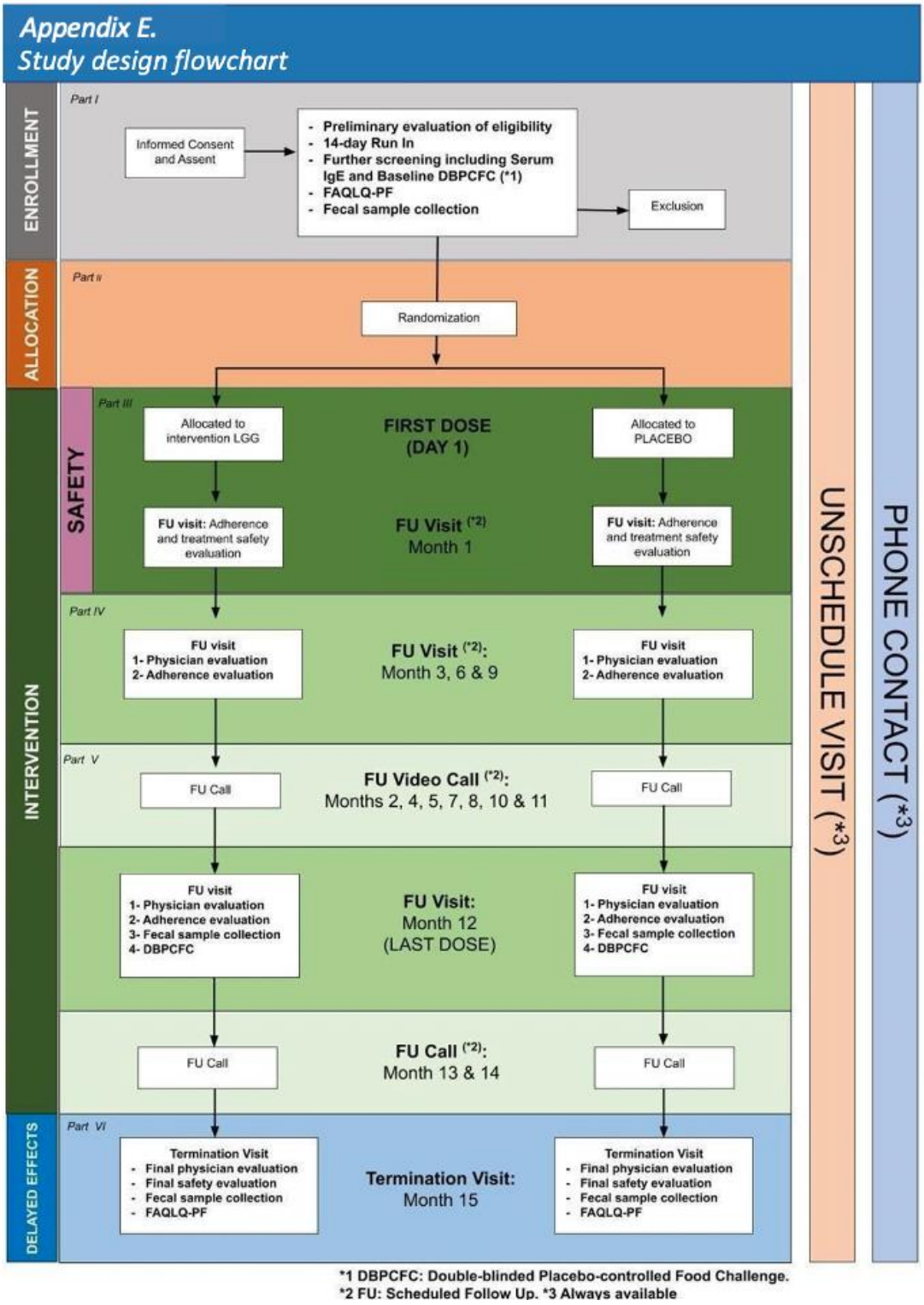
Previous surgery?	Yes ( ) No ( )	Describe:

Family Medical History:

Appendix D.
Participant Timeline

Assessment/ Procedure	-28 D		D0	MONTH (+/- 7 days)													
	D1	D7		1	3	4	5	6	7	8	9	10	11	12 D1	12 D7	13	15
ICF process	X																
Eligible criteria checklist	X		X														
Run In (14 days)	X	X															
Serum Immunoglobulin E (IgE)	X																
Fecal sample collection	X														X		X
DBPCFC	X	X												X	X		
Complete anamnesis			X														
Demographic data collection			X														
FAQLQ-PF			X														X
Web based randomization			X														
Treatment (IWRS) & adherence			X		X			X			X			X			
Followup visit					X			X			X						
Followup call				X		X	X		X	X		X	X			X	
Termination visit																	X

ICF: Informed Consent Form; DBPCFC: Double-blinded Placebo-controlled Food Challenge; FAQLQ-PF- Food Allergy Quality of Life Questionnaire - Parent Form; IWRS- Interactive Web Response System; -28D: Upto 28 days before randomization; D0: Randomization and baseline; D1/D7: Day 1/7



*1 DBPCFC: Double-blinded Placebo-controlled Food Challenge.
*2 FU: Scheduled Follow Up. *3 Always available

Appendix F.
Food Allergy Quality of Life Questionnaire (FAQLQ-PF)

Because of food allergy, my child feels.....

1 Worried about food

2 Different from other children

3 Frustrated by dietary restrictions

4 Afraid to try unfamiliar foods

5 Concerned that I am worried that he/she will have a reaction to food

Because of food allergy, my child.....

6 Experiences physical distress

7 Experiences emotional distress

8 Has a lack of variety in his her diet

Because of food allergy, my child has been negatively affected by.....

9 Receiving more attention more attention than other children of his/her age

10 Having to grow up more quickly than other children of his/her age

11 His/her environment being more restricted than other children of his/her age

Because of food allergy, my child's social environment is restricted because of limitations on.....

12 Restaurants we can safely go to as a family

13 Holiday destinations we can safely go to as a family

Because of food allergy, my child's ability to take part has been limited.....

14 In social activities in other people's houses (*sleepovers, parties, playtime*)

15 In preschool/school events involving food (*class parties/treats/lunchtime*)

Because of food allergy, my child feels.....

16 Worried when going to unfamiliar places

17 Concerned that he/she must always be cautious about food

18 'Left out' in activities involving food

19 Upset that family social outings have been restricted by the need to plan ahead.

20 Concerned about accidentally eating an ingredient to which he/she is allergic

21 Worried when eating with unfamiliar adults/children

22 Frustrated by social restrictions

Because of food allergy, my child.....

23 Is more worried in general than other children of his/her age

24 Is more cautious in general than other children of his/her age

25 Is not as confident as other children of his/her age in social situations

26 Wishes his/her food allergy would go away

Appendix G.1.
PRACTALL Scoring System

Adapted from (Sampson et al., 2012)

I. SKIN

A. Erythematous Rash- % area involved_____

B. Pruritus
0 = Absent
1 = Mild, occasional scratching
2 = Moderate -scratching continuously for > 2 minutes at a time
3 = Severe – hard continuous scratching – excoriations

C. Urticaria/Angioedema
0 = Absent
1 = Mild – < 3 hives, or mild lip edema
2 = Moderate - < 10 hives but >3, or significant lip or face edema
3 = Severe – generalized involvement

D. Rash
0 = Absent
1 = Mild – few areas of faint erythema
2 = Moderate – areas of erythema
3 = Severe – generalized marked erythema (>50%)

II. UPPER RESPIRATORY

A. Sneezing/Itching
0 = Absent
1 = Mild – rare bursts, occasional sniffing
2 = Moderate – bursts < 10, intermittent rubbing of nose, and/or eyes or frequent sniffing
3 = Severe – continuous rubbing of nose and/or eyes, periocular swelling and/or long bursts of sneezing, persistent rhinorrhea

III. LOWER RESPIRATORY

A. Wheezing
0= Absent
1 = Mild – expiratory wheezing to auscultation
2 = Moderate – inspiratory and expiratory wheezing
3 = Severe – use of accessory muscles, audible wheezing

B. Laryngeal
0= Absent
1 = Mild – >3 discrete episodes of throat clearing or cough, or persistent throat tightness/pain
2 = Moderate – hoarseness, frequent dry cough
3 = Severe – stridor

IV. GASTROINTESTINAL

A. Subjective Complaints
0 = Absent
1 = Mild–complaints of nausea or abdominal pain, itchy mouth/throat
2 = Moderate – frequent c/o nausea or pain with normal activity
3 = Severe - notably distressed due to GI symptoms with decreased activity

B. Objective Complaints
0 = Absent
1 = Mild – 1 episode of emesis or diarrhea
2 = Moderate – 2-3 episodes of emesis or diarrhea or 1 of each
3 = Severe – >3 episodes of emesis or diarrhea or 2 of each

V. CARDIOVASCULAR/NEUROLOGIC

0 = normal heart rate or BP for age/baseline
1 = mild-subjective response (weak, dizzy), or tachycardia
2 = moderate-drop in blood pressure and/or >20% from baseline, or significant change in mental status.
3 = severe-cardiovascular collapse, signs of impaired circulation (unconscious)

LEGEND:

GREEN:
- Not usually an indication to alter dosing.
- Not generally sufficient to consider a challenge positive.

RED:
- Objective symptoms likely to indicate a true reaction
- Usually an indication to stop dosing.

Orange (scores increasing to orange):
- Caution, dosing could proceed, be delayed, have a dose repeated rather than escalated.
- If clinically indicated, dosing is stopped.
- Symptoms that recur on 3 doses, or persist (e.g., 40 minutes) are more likely indicative of a reaction than when such symptoms are transient and not reproducible.
- 3 or more scoring areas in orange more likely represent a true response.

Appendix G.2.
Scoring of Allergic Reactions

Adapted from (Sampson et al., 2012)

Category	Symptom	Grade			
Skin	Erythematous Rash (% Area Involved)	0	1	2	3
	Pruritus	0	1	2	3
	Urticaria/Angioedema	0	1	2	3
	Rash	0	1	2	3
Upper Respiratory	Sneezing/Itching	0	1	2	3
		0	1	2	3
		0	1	2	3
		0	1	2	3
Lower Respiratory	Wheezing	0	1	2	3
	Laryngeal	0	1	2	3
Gastrointestinal	Subjective Complaints	0	1	2	3
	Objective Complaints	0	1	2	3
Cardiovascular/ Neurologic	Heart rate or BP for age/baseline	0	1	2	3

Appendix G.3.
DBPCFC Data Collection Format

Patient ID *Discrete Continuous Variable*	**DBPCFC week** Week 1=1 Week 2=2 *Categorical Variable*	**Sex** Female=0 Male=1 Other=2 *Categorical Variable*	**Age at DBPCFC (years)** *Continuous Variable*	**Results of DBPCFC** Positive=1 Negative=0 Inconclusive=2 *Categorical Variable*	**CM dose that triggered symptoms during DBPCFC (mL)** *Continuous Variable*	**Time of ocurrence of symptoms after CM dose during DBPCFC (min)** *Continuous Variable*

Scoring of Allergic Reactions (ARs) (Sampson et al., 2012)

Grade of Severity of ARs (Sampson et al., 2012)	
	Absent=0; Mild=1; Moderate=2; Severe=3 (Ordinal Categorical Variable)
	Absent=0; Green=1; Orange=2; Red=3 (Categorical Variable)

Dermatologic				**Upper Respiratory**	**Lower Respiratory**		**Gastrointestinal**		**Cardio-vascular/ Neurologic**
Erythem-atous Rash (% Area Involved) *Continuous Variable*	**Pruritus** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Urticaria /Angio -edema** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Rash** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Sneezing /Itching** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Wheezing** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Laryngeal** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Subjective Complaints** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Objective Complaints** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Abnormal heart rate or BP for age/baseline** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*

Delayed ARs *if applicable* (Sampson et al., 2012) *for more than one episode, use a new table.*

Duration (min) >20 minutes after last dose *Continuous Variable*	

Dermatologic				**Upper Respiratory**	**Lower Respiratory**		**Gastrointestinal**		**Cardio-vascular/ Neurologic**
Erythem-atous Rash (% Area Involved) *Continuous Variable*	**Pruritus** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Urticaria /Angio -edema** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Rash** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Sneezing /Itching** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Wheezing** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Laryngeal** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Subjective Complaints** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Objective Complaints** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*	**Abnormal heart rate or BP for age/baseline** Absent =0 Mild =1 Moderate =2 Severe =3 *Ordinal Categorical Variable*

Appendix H

VISIT FORMAT

Date:

Name:

Contact:

Age:

Sex:

D1 Intervention:

Side effects: Yes ( ) No ( )

Dates of food challenge:

Documented Allergic Reactions				
Skin	Upper Respiratory	Lower Respiratory	Gastrointestinal/Neurological	Erythematous
Pruritus	Urticaria/Angioedema	Sneezing/Itching	Wheezing	Laryngeal

Appendix I1. ***Data Management***
Physical copies of the informed consent forms and any applicable surveys provided, such as those that will be applied for safety monitoring during the trial, will be stored onsite at the testing center with copies kept at a backup location.
All applicable research data will be entered and stored electronically at the clinical center with a backup copy stored off-site.
Participant forms and files will be stored in a categorized manner and kept in storage for no less than three years after the trial's culmination.
A subset of the original paper forms and trial data from each participating site will be queried and reviewed by independent observers to ensure quality control and compliance with ethical and legal standards.
All clinical information will be entered in an electronic format. Any data recorded in paper format will be converted to electronic as possible. An independent data-entry firm will be used for this purpose, ensuring the anonymity of the data in question. A subset of this converted data shall be queried at random and checked to ensure faithful and consistent reproduction of the inherent information and a sampling of the rate of any possible errors.
The central database shall be kept secure with password protections that shall be changed regularly. Backup hard drives shall be stored at the trial center and kept secure under lock and key in regulation steel cabinets. Regular backups shall be carried out once a month. A second set of computer backups shall be kept off-site in a climate-controlled environment. Only the steering committee members shall be authorized registered access to the hard drives. The electronic database shall be available in a two-tier system, i.e., for viewing purposes only for a subset of the involved and for the researchers' editing purposes. Any edits or modifications to the database shall also be logged under the applicable user IDs.
Any outside requests for release or editing of the information shall be delivered to the Information Technology representative assigned by the trial lead, who shall be tasked with responding in a time-sensitive and appropriate manner after conferring with the steering committee and in compliance with applicable ethical and legal bounds. Any requests for correction of errors shall be reviewed similarly - any approved edits must be made to both the electronic and paper documents with a record kept of the changes made.
The center shall ensure that its operating environment meets the study's minimum hardware and software requirements.

Appendix I2. ***Data Monitoring***
Given the nature of this trial as involving pediatric participants and their exposure to potentially harmful allergens, this trial qualifies for the oversight of an independent Data Monitoring Committee (DMC).
The DMC shall be established as a body independent of the study organizers. It shall consist of five members, including a representative of the hospital ethics committee, a hospital research committee representative, an information technology specialist, a statistician, and a trial lead representative. All members shall be required to meet a minimum standard of data and information technology competency.
The committee shall be provided with access to the central database in a view-only format along with any other documents. The DMC's primary role should be to determine, through periodic review, whether there are any errors or lapses in trial safety or data management and whether the accumulating data have affected clinical equipoise to the degree that a recommendation to modify or discontinue the trial must be made.