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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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Abstract:

Background: *IgE-mediated reactions constitute 70% of cow's milk-induced allergic reactions (AR). There is no established treatment. Recent evidence suggests that immunomodulation with probiotics represents a safe novel strategy, influencing immunity and inducing tolerance to milk protein antigens by action on human gut microbiota. Current evidence focuses most on the infant population. Objective: We will study the impact of Lactobacillus rhamnosus GG (LGG), compared to placebo, as an effective agent to improve ARs upon exposure to cow's milk in children from 5 to 10 years of age.*

Methods: *This will be a phase II, single-center, randomized, double-blinded, placebo-controlled study, where a total of 200 participants will be treated for 12 months, with either; LGG or placebo, randomly allocated at 1:1. A double-blinded placebo-controlled food challenge (DBPCFC) with cow's milk will be used before and after the intervention. Results will be graded using the PRACTALL scoring system. The primary outcome will be binary – 'passing' by the absence of any AR, or a decrease from the baseline results or 'not passing' the DBPCFC after treatment. Secondary outcomes will include covariate adjustment and subgroup analysis by affected body systems and severity of ARs. Secondary analyses will include a comparison of the proportions of the taxonomic composition of gut microbiota, and quality of life, with baseline measurements.*

Conclusion: *This trial will contribute to filling knowledge gaps about cow's milk allergy management using LGG in this specific population as an affordable and accessible non-pharmacological agent with few recorded side effects. If proven to be efficacious, it has the potential to decrease the worldwide prevalence of CMA and the resulting systemic, familial, and personal burdens.*

Keywords: *IgE-mediated cow milk allergy; cow milk allergy; Lactobacillus rhamnosus GG; LGG; double-blinded placebo-controlled food challenge; PRACTALL Scoring System.*

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Abbreviations
AR: Allergic Reaction
LGG: Lactobacillus rhamnosus GG
DBPCFC: Double-blinded Placebo-controlled Food Challenge
CMA: Cow's Milk Allergy
Non-IgE-CMA: Non-IgE-mediated Cow's Milk Allergy
IgE-CMA: IgE-mediated Cow's Milk Allergy
QoL: Quality of Life
FA: Food Allergy
GM: Gut Microbiota
FC: Food Challenge

INTRODUCTION

CMA is a pathological immune system reaction triggered by milk protein antigen, usually diagnosed early in childhood (Carbonell Oriel & Wang, 2019). It has a worldwide prevalence that can range between 0.5-9% in children (Canani et al., 2017; Hochwallner et al., 2014); approximately one out of four children with CMA in the U.S. fall in the group age of 6 to 10 years (Warren et al., 2013).

ARs might range from mild to life-threatening, such as anaphylaxis (Yu et al., 2016). Non-IgE-CMA presents with a mild and short course; however, IgE-CMA constitutes 70% of cow's milk-induced ARs and may lead to delayed development of tolerance and even persist through adulthood (Canani et al., 2017; Saarinen et al., 2005). CMA represents up to 19% of the cases of all food-induced anaphylaxis in children less than 16 years of age (Hochwallner et al., 2014; Kattan et al., 2011).

IgE-CMA also leads to a substantially decreased QoL (Antolín-Amérigo et al., 2016), with a significant financial burden, social constraints, and emotional demands. Among children with more than one type of FA, parents reported that CMA was the most socially limiting, the one that required more planning and caused more anxiety (Abrams et al., 2020).

There is no cure for this type of allergy. Continuous symptomatic pharmacological management is unfeasible due to adverse effects. Current management is avoidance of cow's milk (Canani et al., 2019). Recently, more active non-pharmacological approaches have emerged, such as immunotherapy, including the use of probiotics (Licari et al., 2019). Probiotics are living microorganisms that confer a health benefit to the host (Paparo et al., 2019), inhibiting pathogens through direct action on the commensal microbiota, the assemblage of microorganisms present in a specific environment (Marchesi & Ravel, 2015; Segers & Lebeer, 2014). Studies have identified a critical role of human gut microbiota in modulating host immune responses by influencing innate and adaptive immunity (Ho & Bunyavanich, 2018; Lee et al., 2020) (**Appendix A.1**).

IgE-CMA mechanisms involve humoral [IgE allergen-specific antibody] and cellular immunity [regulatory T cells] (Sicherer & Sampson, 2018), including aberrant T helper type 2 cell response from a dysbiotic modification in the microbiota (Aitoro et al., 2017). Immunomodulation with probiotics represents a novel strategy, where gut bioactive peptides induce tolerance to milk proteins (**Appendix A.2**). One of the most used probiotics is LGG [Lactobacillus rhamnosus GG], a safe modulator in allergic diseases, acting on the inflammatory response by increasing cellular modulation of complement receptors (Segers & Lebeer, 2014) (**Appendix A.3**).

Improvement in the severity of ARs in infants using hydrolyzed whey formula with LGG suggests its efficacy (Canani et al., 2017). However, results were not adequately robust to provide a formal recommendation (Majamaa & Isolauri, 1997; Segers & Lebeer, 2014). Scalabrin et al. (2017) also demonstrated its efficacy in children below five years of age. Additionally, 80-90% of the children's CMA have been shown to resolve within the fifth year of life (Caffarelli et al. 2010). Therefore, we are focusing on children with this resistant type of allergy.

The age group of 5 to 10 years is still largely unexplored. We plan to conduct a trial in children in this age range, with a confirmed IgE-CMA diagnosis, to study the occurrence of ARs upon exposure to cow's milk after 12 months of treatment with LGG. We hypothesize that there will be a significant improvement in ARs, measured by the DBPCFC, compared to placebo.

METHODS

Trial Design

This is a phase II, single-center, randomized, double-blinded, placebo-controlled study, designed to comply with the required ethical principles and all the International Conference on Harmonization principles, to be conducted in an academic urban tertiary hospital in the United States of America. Parental consent and child assent will be acquired before recruitment. (**Appendix B1-B2**)

All participants who agree to enroll will be screened (see **Figure 1** 'Eligibility Criteria' and

Eligibility Criteria

Inclusion Criteria	
5 - 10 years age	Clinical history of CMA, collected in anamnesis
Parental Consent, Participant Assent and availability of care provider or responsible adult	
Avoidance of consumption of cow-milk or derivatives for at least three months before recruitment - and agreement to continue avoidance for the length of study participation	
Other Known FAs: Agreement to avoid consumption of offending allergens for the length of study participation	
Demonstration of strict protocol adherence during run-in period	
Positive Milk-specific Serum Immunoglobulin E (IgE) Test	Positive DBPCFC result (≤300 mg of milk protein)

Exclusion Criteria	
Allergy to LGG, soy and its derivatives, and components of the gummies	
Administration of any pre- or probiotics up to four weeks prior to enrollment	
Ongoing treatment in the moment of enrollment or the preceding month, with any antibiotic or steroid	
Ongoing immunotherapy	
Central venous catheter-in-situ	Severe or uncontrolled asthma
Malformations of the respiratory tract	Chronic pulmonary diseases
Malformations of the gastrointestinal tract	Deglutition problems
Immunocompromise: any malignancy, HIV, Chemo/radiotherapy	Chronic inflammatory bowel diseases
Non–CMA-related atopic eczema	Celiac disease
Eosinophilic disorders of the gastrointestinal tract	Cystic fibrosis
Active tuberculosis	Metabolic diseases
Congenital cardiac defects	Autoimmune diseases

Figure 1. Eligibility Criteria

Appendix C 'Medical History Format') after successfully completing a 14-day run-in period with placebo (see 'Adherence' below). As part of the inclusion criteria, a DBPCFC (see below) will be done to confirm the IgE-CMA diagnosis. It will also be used to record baseline data on severity and affected organ systems. (See **Appendix D** 'Timeline')

Participants will be randomly allocated at a 1:1 ratio into two treatment arms – LGG or placebo – with a once-daily regimen for 12 months. (See 'Randomization' below)

At the end of the 12 months, a second DBPCFC will be administered to both arms to compare results with the baseline data of each arm.

A fecal sample will be collected at the baseline to record the composition of the GM using 16S rRNA gene sequencing, and then it will be compared with the composition of a second sample collected at the end of treatment at 12 months and a third sample collected at 15 months – to observe the modification in the GM over time, without treatment (see **Appendix E** for study design flowchart).

Responses to a Food Allergy Quality of Life Questionnaire - Parent Form [FAQLQ-PF] (**Appendix F**) will be collected before treatment and compared to another collected at 15 months.

Double-blinded Placebo-controlled Food Challenge [DBPCFC]: A FC is a procedure used to diagnose, monitor for resolution of, or identify the threshold of responsiveness to a suspected allergen by its oral administration in a controlled and standardized setting (Carbonell Oriel & Wang, 2019). FAs are known to have a psychogenic component – a patient with a history of moderate/severe allergy may elicit a subjective response to the allergen even when the pathophysiologic pathway is not activated. The physician assessing the FC may also induce an interpretation bias. The DBPCFC has a specific validated methodology designed to negate these effects and is considered the gold standard for diagnosing FA (Gushken et al., 2013).

Our proposed DBPCFC will consist of two FCs. The first will be conducted with either cow's milk or sham milk; the second will be conducted one week later, with the converse. The order of administration of preparation – cow's milk and sham milk – will be randomly assigned during the initial phase of each

DBPCFC with a computer-generated list using REDCap at a 1:1 ratio.

After at least four hours of fasting, the FCs will be administered during the morning. Following design suggestions by Calvani et al. (2019), guardians will be advised to interrupt the participants' use of any antihistamine, proton-pump inhibitor, or beta-blocker for 72 hours before and during the FCs, as these medications may confound the results. The cow's milk group will receive incremental doses of pasteurized lactose-free cow's milk – 0.1, 0.3, 1, 3, 10, 30, and 100 mL – diluted in 50 mL of soymilk (Canani et al., 2017). The sham-milk group will receive equal incremental doses of water, diluted in 50 mL of soymilk. There will be 20-minute intervals between doses. The preparations will be given in 200 mL sealed opaque cups with opaque straws and identical in weight, appearance, and material. The soymilk vehicle's distinct organoleptic properties will mask the negligible variation created by the cow's milk or water. Patients will be followed for 30 minutes after the end of the FC to guarantee their safety. The response to each dose will be assessed by the PRACTALL Scoring System (**Appendix G.1**). It is a scoring system detailed in the PRACTALL Consensus Report, used in IgE-mediated allergies to determine the degree of response in target organs and their baseline changes. ARs are detailed in severity on a scale from 0 to 3, with colors black, green, orange, and red (Sampson et al., 2012).

The challenge will be stopped at the dose following which any symptom reaches a 'red' grade or two symptoms from different categories reach an 'orange' grade (**Appendix G.2**) (Sampson et al., 2012). The dose, category, and grade will be recorded (**Appendix G.3**). The participant will be discharged after the symptoms are resolved. If no reaction occurs until the last dose of a challenge, participants will be discharged after three hours of observation. Guardians will be instructed to report any delayed symptoms to correct initial false-negative results.

All participants will be placed with a peripheral venous catheter in case of any severe AR during the FCs, which will be conducted in the outpatient clinic of a tertiary hospital. All appropriate resources will be available, including antihistamines, epinephrine, tracheal intubation equipment, and a defibrillator (Gushken et al., 2013). Different physicians will be involved on different days to avoid interpretation bias and inadvertent unblinding; all will have emergency management expertise. Our eligibility criteria are presented in **Figure 1**.

Recruitment Strategy

After the protocol is granted permission to start, we will disseminate it within the pediatric outpatient offices and inpatient departments of the study center and its staff to enable convenience sampling using the patient database. We will also encourage disclosure between physicians, allergy clinics, and pediatricians in the city and neighboring regions. Patients reached by this strategy will be screened and added to the trial, following a convenience strategy. To incentivize enrollment, participants who complete the trial in the placebo arm will be offered the active treatment for one year, free of cost, if proven to be efficacious. The participants will be randomized within 28 days from the day the informed consent has been signed. We intend to enroll at least 40 participants every month.

Randomization

Participants will be allocated by blocked randomization with variable block sizes – 6-4 – at a 1:1 allocation ratio by an internet-based computer-generated sequence, using REDCap. For the trial intervention, an unblinded nutritionist will be in charge of the randomization list and send the numbered sealed containers to the research team.

For the DBPCFCs, an unblinded dietician will be in charge of the randomization list and preparations and will deliver the numbered sealed disposable cups to the FC staff before each FC.

Blinding

Participants, guardians, researchers, follow-up physicians, DBPCFC physicians and staff, and statisticians will be blinded to the intervention and the DBPCFC allergen for the entire trial length.

The interventions {gummies; see 'Interventions' below} will have identical organoleptic properties and packaging.

REDCap will be used to collect the data from follow-up visits and the post-treatment DBPCFC. The first DBPCFC data will be unblinded to the researchers to be considered in the inclusion criteria.

Emergency unblinding

Unblinding will be carried out when the participant's safety is at risk, and this knowledge is required for emergency treatment. If stopping the intervention is feasible for the participant's care, blinding will be preserved. A record of the request and its justification

will be kept. Unblinded data will not be eliminated from statistical analyses.

The DBPCFC will be administered in a controlled environment. Treatment of incidental ARs, including acute/emergent management, will not require knowledge of allocation.

Interventions

The interventions will be in the form of gummies. All gummies will be composed of water, isomalt, maltitol, citrus pectin, black carrot extract, beta carotene, natural orange or strawberry flavor, citric acid, and sodium citrate. Active treatment gummies will also contain 2.0 x 10^10 CFU [Colony Forming Unit] of LGG. The dosage selected was based on previous studies of LGG performed for FA – Canani et al. (2017); Gushken et al. (2013); Pohjavuori et al. (2004); Tang et al. (2015).

Adherence

The LGG requires no preparation, making it easier for the guardians. Gummies will be provided in containers with child-proof lids in a monthly supply, making adherence monitoring easy and will prevent accidental ingestion and wastage. Guardians will also be oriented to fill a daily record of the date and time of each administration. In the first visit after randomization, we will orientate the guardians and children regarding diet and how to avoid allergens in both groups.

Follow-up will include periodic video/phone calls and visits (see **Appendix E and H** 'Visit Format'). The guardians' travel and parking expenses will be reimbursed. Participants and their guardians will be counseled about adherence and the importance of disclosing any deviation. They will be reassured about disclosing minor deviations and warned about significant deviations and how that might ultimately affect the trial and its results.

The security of the supplied interventions will also be ascertained during the follow-up interactions, and the guardians will be urged to pick up a replacement supply in case applicable. A note of the incident will be made. A count of supplement intake and product remaining will be established during every visit.

For the DBPCFC, the participants will be allowed to choose from two artificial flavors of the soymilk diluent – strawberry or passion fruit. Children will have access to a designated supervised play area. Guardians will be offered reading material and be given comfortable seating; both will be offered a complimentary meal to ensure attendance for both days of the DBPCFC.

A two-week run-in phase will be used to minimize the drop-out rate. Only participants who can demonstrate 100% of intervention adherence will move on to further screening and subsequent randomization.

Discontinuation Criteria and Safety

Probiotics are generally considered safe and well-tolerated (Segers & Lebeer, 2014). Some adverse events like mild abdominal cramping, diarrhea, or flatulence may occur for a few days after treatment initiation. Participants and guardians will be counseled about mild, transient symptoms to discourage dropping out in such cases. In all situations, adverse events will be actively asked in the follow-up visits.

In the following situations, the intervention may be discontinued, and the participant terminated from the trial:

- Adverse reactions to treatment that last more than one week after initiation or are severe.
- Major surgery, hospitalization with central venous access.
- Upon request or due to logistic difficulties – e.g., moving.

Note: If a participant receives any antibiotic during the study, this information will be recorded – type, dosage, reason, and duration. The participants will not be excluded from the trial.

To preserve uniformity of exposure to the intervention, we shall not implement any modification criteria.

Data Management and Monitoring

See **Appendices I1 and I2.**

Outcomes

Primary Outcome

Our primary outcome will be Binary, as 'passing' or 'not passing' the DBPCFC at the end of the 12-month treatment. Passing will be defined by the absence of any AR of grade 1 or more, or a decrease from the baseline results to the 'green' categories according to the PRACTALL Scoring System (**Appendix G.2**).

Secondary Outcomes

Secondary outcomes will be a comparison between groups in:

- Taxonomic composition of GM from fecal samples, as proportions – continuous variables.
- QoL – continuous variables.

Statistics

Sample Size Calculation

We aim to detect a difference of 20% in the improvement of tolerance in ARs in the LGG group versus the placebo group (Canani et al., 2017), with a power of 80% and an α level of 0.05. Assuming a drop-out rate of 10% at follow-up, we have 100 participants per group with a total sample of 200 participants.

Statistical Analyses

(using Stata Statistical Software: Release 16 (College Station, TX: StataCorp LLC))

Continuous baseline variables will be measured by mean and standard deviation, if the distribution is normal, and median and interquartile range, if it is not normal. Categorical baseline variables will be measured by frequency and percentiles. We assume a nondirectional hypothesis.

For the binary primary outcome, we shall conduct a Chi-square test to measure the intervention's independent association with the change in the proportion of participants who pass the second DBPCFC.

We have also planned a logistic regression model as a secondary analysis to adjust the intervention by the affected body system and the severity of ARs as in the PRACTALL score. Moreover, we plan to use the logistic regression model to estimate a subgroup effect for system and severity at baseline by including an interaction between the trial arm and each of the variables.

For the secondary outcome of taxonomic proportions of different microbiota species, we shall use a linear mixed model to assess the modifications in the proportions throughout the trial, that is, at baseline, 12 months, and 15 months.

We shall use a t-test to compare the change in the QoL score between groups. Finally, we will assume significance in a p-value ≤ 0.05.

Missing Data

We will adopt an intention-to-treat approach. Multiple imputation methods will be used to estimate replacement values based on each participant's baseline characteristics.

DISCUSSION

CMA is a highly prevalent worldwide issue, with a large pediatric population and no available cure. It is associated with physical health implications, mental health, and QoL, also a significant financial burden.

The study of GM is a current global hot topic. There have also been multiple studies evaluating the effect of probiotics, such as LGG, on CMA (Basturk et al., 2020; Scalabrin et al., 2017). To our knowledge, the age range of 5 to 10 years has not been studied, and most of the evidence focuses on younger populations. We also understand that establishing evidence in this age range will impact healthcare systems, as similar studies in younger populations have demonstrated a positive impact on healthcare costs (Guest et al., 2016; Guest & Singh, 2019).

There is also a dearth of scientific literature on the association of the GM's composition with the occurrence of CMA manifestations, as well as on the temporal association of the use of LGG with taxonomic changes in the GM.

The primary endpoint is the occurrence of ARs in the DBPCFC after 12 months of intervention - to be treated as a binary variable. We have chosen this endpoint to avoid the ambiguity that may arise from defining what constitutes an improvement, although this may lead to a negative result.

The trial duration may constitute a limitation since most previous trials have spanned two to three years. However, there is robust evidence that supports the effect of the LGG after 12 months in a younger population (Canani et al., 2017). Evidence to declare efficacy is scarce in the age range we intend to assess and demands a shorter duration Phase II trial.

This trial has some commendable attributes and contributes to filling some significant knowledge gaps. The methodology is robust enough to generate a sizable amount of data on the efficacy of LGG for IgE-CMA management, in addition to clinically relevant secondary outcomes. The array of statistical tools to be used is simple yet powerful while properly managing potential missing data. For these reasons, we believe this trial will bring relevant information even if the result (from the primary endpoint) is deemed statistically negative.

In conclusion, we attempt to address a critical barrier for CMA management in children, using LGG as a safe, affordable, and accessible non-pharmacological agent. It is an innovative alternative that, if proven effective, will improve the lives of affected children and positively impact worldwide prevalence and the resulting healthcare burden and costs. However, as a phase II single-center trial, it will not be conclusive evidence. Therefore, more generalizable and bigger studies will need to be performed in the future. Our

study is a great step in the way of this translational science.

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Conflict of interest

The authors declare that this research protocol was developed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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