Food and Chemical Toxicology 190 (2024) 114817

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Workshop report: A study roadmap to evaluate the safety of recombinant human lactoferrin expressed in *Komagataella phaffii* intended as an ingredient in conventional foods – Recommendations of a scientific expert panel

Carrie-Anne Malinczak [a], Leigh Ann Burns Naas [b], Anthony Clark [a], Dietrich Conze [c], Michael DiNovi [d], Norbert Kaminski [e], Claire Kruger [c], Bo Lönnerdal [f], Nicholas W. Lukacs [g], Robert Merker [h], Ross Peterson [a,*]

[a] *Helaina Inc. New York, New York, USA*
[b] *Magnolia Consulting Services LLC, Traverse City, MI, USA*
[c] *Spherix Consulting Group, Rockville, MD, USA*
[d] *DiNovi Regulatory Associates LLC, Baltimore, MD, USA*
[e] *Department of Pharmacology & Toxicology, Institute for Integrative Toxicology, Michigan State University, East Lansing, MI, USA*
[f] *Department of Internal Medicine, University of California, Davis, CA, USA*
[g] *Mary H. Weiser Food Allergy Center, University of Michigan, Ann Arbor, MI, USA*
[h] *Independent Consultancy, Washington, DC, USA*

ARTICLE INFO

Handling Editor: Dr. Bryan Delaney

Keywords:
Immune safety
Ingredient risk assessment
Clinical studies
Immunotoxicology
Toxicokinetics

ABSTRACT

Human milk lactoferrin (hmLF) is a glycoprotein with well-known effects on immune function. Helaina Inc. has used a glycoengineered yeast, *Komatagaella phaffii*, to produce recombinant human lactoferrin (Helaina rhLF, Effera™) that is structurally similar to hmLF with intended uses as a food ingredient. However, earlier FDA reviews of rhLF were withdrawn due to insufficient safety data and unanswered safety questions the experts and FDA raised about the immunogenicity/immunotoxicity risks of orally ingested rhLF. Helaina organized a panel of leading scientists to build and vet a safety study roadmap containing the studies and safety endpoints needed to address these questions. Panelists participated in a one-day virtual workshop in June 2023 and ensuing discussions through July 2023. Relevant workshop topics included physicochemical properties of LF, regulatory history of bovine LF and rhLF as food ingredients in the FDA's generally recognized as safe (GRAS) program, and synopses of publicly available studies on the immunogenicity/alloimmunization, immunotoxicology, iron homeostasis, and absorption, distribution, metabolism, and excretion of rhLF. Panelists concluded that the safety study roadmap addresses the unanswered safety questions and the intended safe use of rhLF as a food ingredient for adults and agreed on broad applications of the roadmap to assess the safety and support GRAS of other recombinant milk proteins with immunomodulatory functions.

1. Introduction

Human milk lactoferrin (hmLF) is an iron-binding glycoprotein with well-documented effects on immune function (Hennart et al., 1991; Lönnerdal, 2013; Manzoni, 2016) that make it an appealing food ingredient. However, isolating it from human milk for large-scale commercial production is cost-prohibitive and presents several ethical challenges. Helaina Inc. has produced recombinant human lactoferrin (rhLF, Effera™) at a commercial scale using a genetically engineered strain of yeast, *Komatagaella phaffii* (formerly classified as *Pichia pastoris*), for a range of food applications. Despite the reclassification of *Pichia pastoris (P. pastoris)* to *Komatagaella phaffii (K. phaffii)* in 1995 (Kurtzman, 2005), many recent references in the GRAS notice database

* Corresponding author. 345 Park Avenue S, 5th Floor, New York, NY, 10010-1707, USA.
E-mail addresses: carrie@myhelaina.com (C.-A. Malinczak), leighann@magnoliatox.com (L.A. Burns Naas), anthony.clark@myhelaina.com (A. Clark), dconze@spherixgroup.com (D. Conze), mike28a@21stcenturyoldguy.com (M. DiNovi), kamins11@msu.edu (N. Kaminski), ckruger@spherixgroup.com (C. Kruger), bllonnerdal@ucdavis.edu (B. Lönnerdal), nlukacs@umich.edu (N.W. Lukacs), robertmerker.phd@protonmail.com (R. Merker), ross@myhelaina.com (R. Peterson).

https://doi.org/10.1016/j.fct.2024.114817
Received 9 April 2024; Received in revised form 3 June 2024; Accepted 12 June 2024
Available online 14 June 2024

Abbreviations

ADME	Absorption, distribution, metabolism, excretion
Anti-LF	Anti-lactoferrin
bLF	Bovine lactoferrin
DC	Dendritic cells
DRF	Dose-range-finding
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FOIA	Freedom of Information Act
GRAS	Generally recognized as safe
HLA-DRB1	Human leukocyte antigen-D-related beta chain
hLF	Human lactoferrin
hmLF	Human milk lactoferrin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
LF	Lactoferrin
NOAEL	No observed adverse effect level
NCBI	National Center for Biotechnology Information
OECD	Organization for Economic Co-operation and Development
PBMC	Peripheral blood mononuclear cells
rhLF	Recombinant human lactoferrin
TK	Toxicokinetics

(e.g., soy leghemoglobin (US FDA, 2018) and myoglobin (US FDA, 2021b) and public literature use the *Pichia pastoris* nomenclature. Results from a recently conducted battery of analytical studies indicate that the physical and chemical characteristics of the Helaina rhLF are similar to those of hmLF (Lu et al., 2024).

In 2023, Helaina organized a virtual, one-day workshop with six leading US experts representing diverse backgrounds in immunology, allergy, toxicology/immunotoxicology, nutrition, ingredient risk assessment, and LF chemistry and function. Supplementary Table 1 (Supplementary material) lists the panel members, their affiliations, and areas of expertise. The two objectives of the workshop were to 1) determine the studies and data needed to assess the alloimmunization and immunotoxicity risks of orally ingested rhLF and 2) evaluate Helaina's analytical and safety study framework to determine if it adequately addresses unanswered safety questions related to the function of LF. The output was a study roadmap detailing preclinical and clinical studies and endpoints to evaluate the safety of rhLF as a food ingredient.

2. Regulatory framework to evaluate the safety of novel ingredients – US context

2.1. GRAS safety standard definition of safety – reasonable certainty of no harm

In the US, the GRAS notification process is broadly recognized by industry as the most common mechanism to make the public aware that a company has determined a food ingredient is safe for defined conditions of use and use levels and that qualified experts would agree with that determination. The GRAS provision is an exclusion from the requirement to do a pre-market approval through FDA regulation; however, the company must still meet the strict safety standard for a valid GRAS conclusion. The safety standard for GRAS substances, as defined in 21 CFR 170.3(i), is reasonable certainty in the minds of competent scientists that a substance is not harmful under the intended conditions of use (Office of the Federal RegisterNational Archives and Records Administration, 2023). In a 2023 publication, FDA review scientists described *reasonable certainty of no harm* as a sensible safety standard, considering that absolute certainty of no harm is nearly impossible to establish scientifically for any food ingredient (Morissette et al., 2023). For the uses of a substance to be GRAS, safety must be demonstrated through scientific procedures or history of use and, further, that there is general recognition among qualified experts that those uses are safe. The pivotal safety data must be generally available and widely accepted. The term *general availability* implies the data are published in peer-reviewed scientific journals, scientific reports, or other scientific publications; *general acceptance* refers to the consensus, but not unanimity, among qualified scientific experts regarding the safety of the substance. Information supporting a GRAS conclusion must satisfy these components to meet the safety standard of reasonable certainty of no harm and the general acceptance criteria. An expert panel is one mechanism a proponent may use to support evidence of general acceptance (US Food and Drug Administration, 2022).

2.2. Prior GRAS notices for LF

Recombinant human lactoferrin has a history in the GRAS program at the FDA. Recombinant human lactoferrin was the subject of three GRAS Notices (US FDA, 2007, 2005, 2004). A comparison of rhLF from transgenic rice and transgenic cows is shown in Supplementary Table 3

Box 1

Unanswered safety questions.

1. *Immunogenicity (Alloimmunization)/Allergenicity/Autoimmunity:* Does ingestion of rhLF lead to a breakdown in tolerance?
2. *Immunotoxicity:* Does ingestion of rhLF lead to immunotoxicity?
3. *Iron homeostasis:* How does ingestion of rhLF affect iron homeostasis?
4. *ADME:* What are the differences in digestion between hmLF and rhLF produced from transgenic *Komatagaella phaffii*?

Summary of the 2008 Expert Panel at Toxicology Forum.

1. Immunogenicity, cross-reactivity, autoimmunity, and allergenicity remain significant concerns.
2. Immunological concerns exist with both oral and parenteral routes of administration.
3. Recombinant proteins are different from their native counterparts, which can present additional safety risks.
4. Assessing the safety of human proteins in animal studies is not a meaningful indicator of safety in humans.

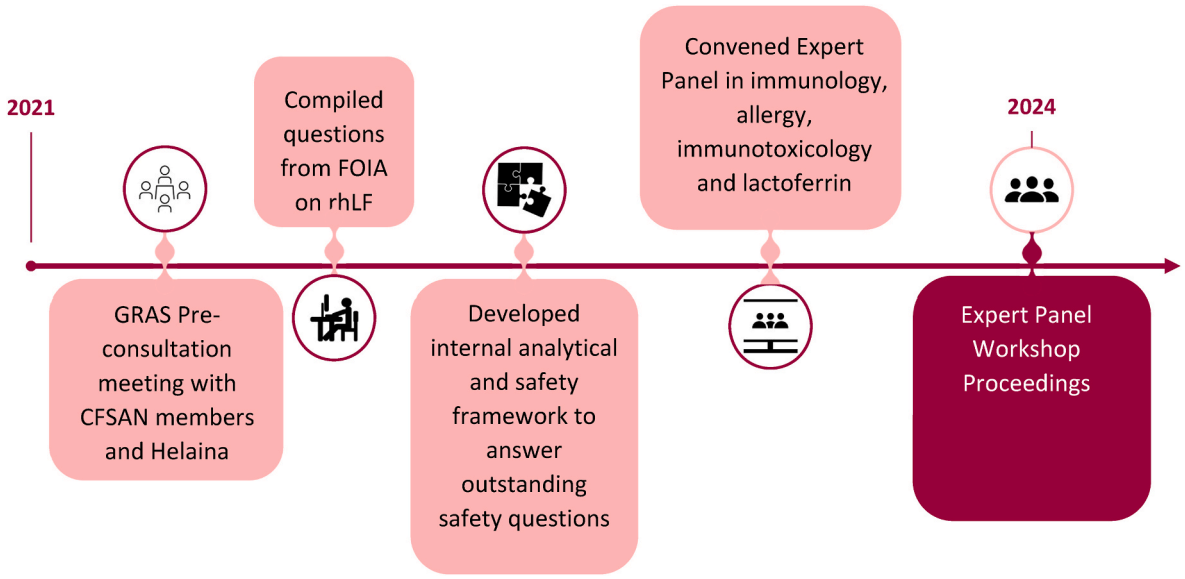

Fig. 1. The timeline of key activities that informed the safety study roadmap of Helaina rhLF to demonstrate *reasonable certainty of no harm*.
GRAS = generally recognized as safe; CFSAN = Center for Food Safety and Applied Nutrition; FOIA = Freedom of Information Act; rhLF = recombinant human lactoferrin; FDA = Food and Drug Administration.

(Fujiyama et al., 2004; Le Parc et al., 2017). These rhLF products showed strong toxicological safety profiles (Appel et al., 2006; Cerven et al., 2008a, 2008b). However, these GRAS Notices were withdrawn due to insufficient data to evaluate endpoints specific to LF functionality coupled with the need for more information about the effects of increased exposure to a protein known to have multiple functions on the immune system. In 2022, the FDA made the correspondence between the agency and each respective notifier of rhLF publicly available through the Freedom of Information Act (FOIA). In addition to safety questions in the present literature identified by Helaina, the FOIA correspondence contained unanswered safety questions raised during these previous notices and a summary of an FDA-moderated session with an expert panel at the 2008 Toxicology Forum on the Use of Recombinant Human Proteins as Food Ingredients (Supplementary Table 2). The primary unanswered safety questions raised by this expert panel in 2008 related to the immune functionality and include the potential for alloimmunization (immunogenicity) and immunotoxicology. Additional considerations raised by FDA in the FOIA documents related to iron homeostasis and absorption, distribution, metabolism, and excretion (ADME) of LF. Following a thorough review of the present-day literature on LF and FOIA correspondence, Helaina concluded that these unanswered questions needed to be addressed to thoroughly evaluate rhLF safety (Box 1).

3. Expert panel method

On June 15, 2023, the experts participated in the panel workshop moderated by Spherix Consulting Group. Helaina Regulatory and Research and Development scientists were also in attendance. Before the workshop, panelists were provided with current scientific background information on LF, Helaina's up-to-date literature assessment of safety information, and the FOIA correspondence on previous rhLF GRAS Notices that included the safety questions previously raised. The timeline and activities central to developing the study roadmap are illustrated in Fig. 1.

During the first half of the workshop, Dr. Dietrich Conze (Spherix) presented current background information on LF. The topics included the structure and function of hmLF, ADME of LF, regulatory history of

Box 2

Descriptions of key terms used by the expert panel.

- Immunogenicity: the ability of the immune system to generate an immune response to a foreign protein (Tao and Raz, 2015)
- Alloimmunization: an immune response triggered when an individual lacking a specific antigen is exposed to this antigen from another human, resulting in an immune response directed at self-proteins (Hendrickson and Tormey, 2016)
- Allergenicity: the ability of an antigen to induce an abnormal immune response (i.e., an overreaction and different from a normal immune response), causing physiological function disorder or tissue damage (Tao and Raz, 2015)
- Immunotoxicity: an adverse effect on immune system structure or function that is caused by chemical or biological compounds resulting from direct or indirect actions and manifesting as either permanent or reversible toxicity (Hinton, 2000)
- Iron homeostasis: physiological mechanisms that regulate dietary absorption, distribution, and concentration of iron in plasma and tissues/organs (Guo et al., 2016)
- Recombinant human lactoferrin: a form of human lactoferrin expressed from transgenic organisms, such as cows, rice, or yeast (e.g., *K. phaffii*), and is structurally and functionally similar to human lactoferrin (Le Parc et al., 2017)
- Immune tolerance: the ability of the immune system to prevent an immune response to self-antigens or against a particular antigen. When tolerance is lost, autoimmune diseases or food allergies/cross-reactivity may occur (Farhangnia and Akbarpour, 2022)

Box 3

Key corroborative preclinical and clinical safety study findings for rhLF presented during the expert panel workshop.

- Animal toxicology studies reported that orally ingested rhLF (from bioengineered rice and cows) was tolerated and showed no toxicity-related changes in safety/tolerability endpoints; the no observed adverse effect levels (NOAEL) were the highest oral doses tested, which ranged between 1000 mg/kg body weight/day and 2000 mg/kg body weight/day (Appel et al., 2006; Cerven et al., 2008a, 2008b).
- Additional animal studies using piglets showed that rhLF (from bioengineered cows) was well-tolerated with no adverse effects (Cooper et al., 2013, 2014a, 2014b; Hu et al., 2012; Li et al., 2014; Zhou et al., 2011)
- In clinical studies conducted in various adult patient populations (e.g., cancer, sepsis) and healthy volunteers, oral ingestion of rhLF (from bioengineered *Aspergillus niger* and rice) was well-tolerated (Digumarti et al., 2011; Guntupalli et al., 2013; Guttner et al., 2003; Hayes et al., 2006, 2010; Jonasch et al., 2008; Laffan et al., 2011; Laskow et al., 2021; Opekun et al., 1999; Parikh et al., 2011; Sortino et al., 2019; Troost et al., 2003; Vincent et al., 2015).

Box 4

Discussion points of the expert panel on rhLF, safety studies, and endpoints to meet the FDA safety standard.

1. The expert panel agreed with the key safety considerations from the expert panel of the 2008 Toxicology Forum moderated by FDA.
2. The ingredient characterization/chemical identity (i.e., full structural characterization) of Helaina rhLF expressed in *Komagataella phaffii* was thorough and complete (Lu et al., 2024).
3. The safety study roadmap is critical to address the key questions raised.
4. Pivotal safety data need to be publicly available through publication in peer-reviewed journals, public meetings, and advisory committees to establish consensus.
5. Animal models are insufficient to assess safety for alloimmunization/immunogenicity endpoints.
6. Evaluate whether the FDA guidance documents from the Center for Drug Evaluation and Research/Center for Biologics Evaluation Research Guidance on Immunogenicity may provide insight in answering safety questions related to immunogenicity.
7. A human clinical trial of sufficient length is suggested to assess the risk of alloimmunization/immunogenicity by evaluating the development of anti-LF antibodies.
8. Human cellular assays with antigen-presenting cells and peripheral blood mononuclear cells (PBMC) are important to assess the immunogenicity potential of rhLF.
9. Unanswered questions related to immune safety should be evaluated in an appropriate rodent study and include immunotoxicological and immunogenicity endpoints.
10. Although in vitro and animal studies show LF has immunogenic potential, their relevance to the immunogenicity of rhLF in humans has not been correlated.
11. Regarding iron saturation, rhLF will take up any free iron, and the contribution of iron from more saturated rhLF is not significant relative to the typical dietary intake of iron. Yet, iron-related parameters should be evaluated in preclinical and clinical studies.
12. The results from the in vivo rat toxicokinetic (TK) study and the in vitro adult digestion study using the standardized INFOGEST protocol (Brodkorb et al., 2019) are sufficient in combination with corroborating published literature regarding ADME of LF to predict rhLF digestion in adult humans; therefore, evaluating ADME of Helaina rhLF in humans is not necessary.

bovine LF (bLF) and rhLF as food ingredients in the GRAS program, GRAS criteria, composition and characterization of Helaina rhLF, and a synopsis of studies on the immunogenicity of rhLF. The goal of the second half of the workshop was to determine if Helaina's safety study plan adequately addressed unanswered safety questions. Following the workshop, expert panelists participated in additional meetings to provide input to refine the final study roadmap. Several essential terms related to the safety studies were mentioned during the workshop and follow-up meetings and are described in Box 2.

4. Summary of preclinical and clinical safety studies presented at the expert panel workshop

This expert panel was convened to review publicly available studies on immunogenicity/alloimmunization, immunotoxicology, and ADME of LF. They were tasked with providing feedback and input on the proposed safety study roadmap to address the unanswered questions and, hence, assess the safety of Helaina rhLF expressed in *Komagataella phaffii*. These scientific experts concluded that performing the agreed-upon studies, assuming positive results, should suffice to meet the GRAS safety standard of reasonable certainty of no harm.

To inform the discussion and the final study roadmap, Dr. Conze presented a comprehensive analysis of corroborative published preclinical and clinical studies related to the safety of rhLF as a food ingredient from various expression systems other than *K. phaffii*. (Vishwanath-Deutsch et al., 2024). The main points from that analysis are shown in Box 3.

5. Expert panel's recommended study roadmap and endpoints to evaluate the safety of recombinant human lactoferrin

Recombinant human lactoferrin is not only a dietary source of iron but also has physicochemical properties that support immune function and is intended for use in conventional foods. Given the intended use of rhLF, the impact of LF on immune function, and the limitations of animal models to assess safety adequately for alloimmunization/immunogenicity endpoints, clinical studies are suggested to evaluate the safety of the increased exposure of rhLF for immune-related endpoints. As such,

Table 1
Planned preclinical and clinical studies to address immune safety questions of alloimmunization/immunogenicity, immunotoxicity, iron homeostasis, and ADME of Helaina rhLF expressed in *K.phaffii*.

Study	Alloimmunization/ immunogenicity	Immunotoxicity	Iron homeostasis	ADME
Allergenicity assessment	✓			
Pepsin digestion	✓			
In vitro adult digestion (INFOGEST Protocol)	✓			✓
14-d DRF rat study		✓	✓	
28-d rat study	✓	✓	✓	✓
Dendritic cell study	✓			
PBMC pilot study	✓			
Ex vivo PBMC T-cell analysis (Epibase®)	✓			
Adult clinical study	✓		✓	

√ = the safety question/s being addressed in each study; FDA = Food and Drug Administration; ADME = absorption, distribution, metabolism, and excretion; rhLF = recombinant human lactoferrin; FARRP = Food Allergy Research and Resource Program (University of Nebraska, US); DRF = dose-range-finding; PBMC = peripheral blood mononuclear cell.

the expert panel emphasized that the study roadmap is critical to address any safety concerns related to the immune system, iron homeostasis, and ADME and affirmed that it adequately did so. A summary of their feedback and recommendations is captured in Box 4. The proposed preclinical studies and clinical study in the roadmap and the safety questions they address are shown in Table 1 and are briefly described here. Human milk LF and bLF will be used as comparative controls in all in vitro studies; however, due to ethical sourcing challenges, the preclinical in vivo and clinical studies where hmLF will not be available, bLF will be used as the active/comparative control. Bovine lactoferrin was selected as a control because of its history of safe use in milk and dairy products and it is currently a GRAS substance and approved as a novel food in the EU for various intended uses including infant formula (EU Commission, 2012b, 2012a; US FDA, 2017, 2014a, 2014b, 2001).

5.1. Allergenicity risk prediction modeling

The allergenicity risk assessments selected for the study roadmap are similar to those used to evaluate the allergenicity potential of soy leghemoglobin expressed by *K. phaffii* (Jin et al., 2018), as well as other proteins notified in the GRAS program. Specifically, these assessments aim to provide allergenicity data that such proteins are not novel and would, therefore, meet general recognition as food ingredients. Assessments include a literature review of peer-reviewed publications to identify studies that reported potential allergenicity of the target protein, LF, the host system, *K. phaffii*, and mannose-containing glycans, bioinformatics of rhLF sequence and host protein allergen assessment, and an in vitro pepsin digestion study.

The NCBI PubMed® database is used to search for peer-reviewed publications that report the allergenicity potential of LF and recombinant proteins from *K. phaffii* and *P. pastoris*, using combinations of species names "*Komagataella phaffii*" and "*Pichia pastoris*" with "clinical allergy," "food allergy," or "IgE binding." Both *K. phaffii* and *P. pastoris* were searched because the naming nomenclatures are found in the literature.

For the bioinformatics sequence allergen assessment, the analyzed amino acid sequences of Helaina rhLF protein and *K. phaffii* residual proteins are used to search the NCBI Protein database for full-length identical sequences. Retrieved sequence searches are used to query the Food Allergy Research and Resource Program (FARRP) AllergyOnline database (University of Nebraska, US) to identify allergenic proteins with similar sequences to rhLF and *K. phaffii*. AllergenOnline is a searchable, peer-reviewed sequence database updated annually to identify proteins that may pose allergenic cross-reactivity risks (Goodman et al., 2016). Notably, *K. phaffii* has been used several times in GRAS notices and is an approved food additive in broiler feed. Importantly, *K. phaffii* proteins are present in several of the notified substances (US FDA, 2023a, 2023b, 2021a, 2021b, 2018, 2006).

Because hmLF and bLF are relatively resistant to digestive proteases, full-length LF and LF fragments may be detectable in feces (Goldman et al., 1990; Hutchens et al., 1991; Kuwata et al., 2001; Spik et al., 1982; Takeuchi et al., 2004; Troost et al., 2001, 2002). Consequently, the incomplete digestion of LF can affect its tolerability and immunogenicity potential. The in vitro digestion study to evaluate the allergenicity potential and digestibility of rhLF is a simple pepsin digestion study (Ofori-Anti et al., 2008; Thomas et al., 2004) (Supplementary Fig. 1). For the simple pepsin digestion study, SDS-PAGE and Western blot analyses are used to detect the presence of surviving LF proteins and peptides after pepsin digestion. Allergenicity potential is determined by comparing the rates of digestion and breakdown of rhLF to those of the comparator proteins, hmLF and bLF.

To further evaluate the digestibility of rhLF, the INFOGEST simulated digestion model (Brodkorb et al., 2019) is utilized. The INFOGEST simulation digestion model is a static digestion model that mimics the sequential process of protein digestion, spanning from the oral phase through the intestinal phase in the presence of appropriate digestive enzymes. Peptidomic analysis (e.g., liquid chromatography and tandem mass spectrometry) and enzyme-linked immunosorbent assays are used to compare quantitative and qualitative end products of rhLF digestion to those of the comparator proteins, hmLF and bLF.

5.2. In vivo immunotoxicology/TK modeling

Two toxicity studies in adult rats are included in the roadmap to confirm the general tolerance of orally ingested Helaina rhLF and evaluate potential immunotoxicological effects. The first is a 14-day dose-range-finding (DRF) toxicity rat study that assesses the effects of repeated daily administration of rhLF at varying doses on general health, toxicity endpoints (e.g., morbidity, mortality, hematology, immune cell phenotyping), and LF detection in serum (Supplementary Fig. 2). Study results are intended to inform the second study, the 28-day immunotoxicity rat study.

The objective of the 28-day immunotoxicity study (US Food and Drug Administration, 2007) is to evaluate the immunotoxicology and immunogenicity potential of orally gavaged rhLF and bLF. The outcomes include general tolerance, immunotoxicology (e.g., T-cell-dependent antibody response, immune cell phenotyping), organ weights, immunogenicity (anti-lactoferrin [anti-LF] antibody detection), iron homeostasis, histology, and TK endpoints (Supplementary Fig. 2).

The panel discussed the relevance of LF immunogenicity studies in in vitro or animal models. Although LF has been previously shown to possess immunogenic potential in these models, relevance to understanding the immunogenicity of rhLF in humans remains to be seen. The expert panelists further acknowledged that the immune safety questions should be evaluated in an appropriate 28-day immunotoxicology study; however, results are not predictive of the potential for alloimmunization in humans. The panel concluded that a clinical study is a more meaningful approach to addressing the alloimmunization/immunogenicity risk. Panelists also agreed that results from the in vivo adult rat TK study, which will provide data on absorption, clearance, and bioavailability to be compared with previous published data, along with the in vitro adult INFOGEST digestion study would be sufficient to predict rhLF digestion

in adult humans, to corroborate the extensive body of literature on the ADME of LF (Vishwanath-Deutsch et al., 2024). Therefore, evaluating the ADME of Helaina rhLF in humans is not necessary.

Because the iron saturation of rhLF derived from *K. phaffii* is typically higher than that of native hmLF, a regulatory expert panelist asked whether such differences would pose any safety concerns. The expert panel indicated LF will take up any free iron and stated that the contribution of iron from a more saturated rhLF is insignificant relative to the typical dietary intake of iron and is highly unlikely to pose a safety concern. For instance, the typical iron content of Helaina rhLF would contribute 3.6% and 1.2% of the Daily Value for 1-3-year-old children and everyone >4 years old, respectively, assuming an exposure at the 90th percentile of the estimated daily intake. Nevertheless, iron parameters will be assessed in all in vivo studies.

5.3. Human immunogenicity modeling

5.3.1. Human cell modeling

In vivo animal models do not necessarily predict alloimmunization/immunogenicity in humans due to species differences. Therefore, in vitro immunogenicity assays using human innate and adaptive immune cells are recommended to detect cell-mediated responses (US Food and Drug Administration, 2021). The steps driving this immunogenic process are 1) priming innate cells, such as dendritic cells (DC), and 2) recruiting T cells to develop a mature immune response, which leads to 3) antibody production by plasma cells.

The panel agreed that human cellular assays with antigen-presenting cells and peripheral blood mononuclear cells (PBMC) are important to assess the immunogenicity potential of rhLF. The methods to evaluate T cell-dependent immune responses include PBMC-based assays. These immunogenicity assays have advantages over animal models, such as using primary cells from various human donors to account for population diversity and targeting different stages of the T-cell-mediated immune response (Bray-French et al., 2021; Nyborg et al., 2016; Siegel et al., 2022). All human primary cellular assays will use rhLF, hmLF, and bLF at levels of 25–50 μg/mL to follow guidelines suggested from Lonza's validated models for immunogenicity testing of immunomodulatory proteins. Additionally, each treatment will include viability assays to ensure a lack of cytotoxicity at the chosen doses. Furthermore, immune stimulation of the cells using positive controls as described below will be used to verify assay performance.

Three human cellular assays are included in the roadmap. The objective of the first study is to measure the activation-induced cytokine production by plasmacytoid DC (pDC, as a surrogate for intestinal DC) and conventional DC (cDC) in response to rhLF, hmLF, and bLF (Perdijk et al., 2018; Spadaro et al., 2008). The cytokine endpoints are shown in Supplementary Fig. 3. To ensure proper activation of the DC, imiquimod and lipopolysaccharide (LPS) will be used as positive controls in the pDC and cDC studies, respectively. The results of this study will provide data about the potential immunogenicity/alloimmunization of rhLF compared to hmLF and bLF.

The second study is a pilot study that aims to investigate the immune stimulation potential of rhLF by exposing PBMC from male and female donors to rhLF, hmLF, and bLF (Nyborg et al., 2016) for 7 days followed by cytokine analysis to include innate and T-cell type cytokines. Positive controls used in this assay will include LPS and KLH to ensure immune stimulation of the PBMC. The outcome of this study will provide insights into the immunogenicity properties of rhLF and its potential for alloimmunization (Supplementary Fig. 4).

The objective of the third study is to examine the immunogenicity risk prediction of rhLF compared to the risk prediction of hmLF and bLF (Jawa et al., 2013; Nyborg et al., 2016), as well as known immunogenic positive controls (e.g. LPS, KLH) using Lonza's validated Epibase® model (Nyborg et al., 2016). This analysis uses ex vivo PBMC from 30 healthy donors to ensure the cells contain the most common HLA-DRB1 alleles in the global population. The HLA-DRB1 alleles are critical for immune recognition and stimulation, and different alleles have varying levels of sensitivity; by controlling for this to get coverage of the most common alleles provides a strong prediction for how the global population may react. Several endpoints are assessed, including the proliferation of various immune cells (e.g. $CD4^+$ T cells) and the production of multiple T-cell specific cytokines (Supplementary Fig. 4) after 7 days of exposure.

5.3.2. Clinical studies

Data on the safety, absorption, and tolerance of ingested rhLF derived from transgenic rice or *Aspergillus niger* var. *awamori* from several clinical studies in patients with acute or chronic conditions (e.g., sepsis, cancer) and healthy volunteers has recently been summarized (Vishwanath-Deutsch et al., 2024). These studies reported that rhLF was well tolerated (Digumarti et al., 2011; Guntupalli et al., 2013; Guttner et al., 2003; Hayes et al., 2006, 2010; Jonasch et al., 2008; Laffan et al., 2011; Laskow et al., 2021; Opekun et al., 1999; Parikh et al., 2011; Sherman et al., 2016; Sortino et al., 2019; Troost et al., 2003; Vincent et al., 2015; Zavaleta et al., 2007). Notably, one study also reported that ingested rhLF derived from transgenic rice did not affect the levels of serum proinflammatory cytokines (Sortino et al., 2019), suggesting that rhLF does not induce systemic inflammation. However, randomized controlled studies are needed to assess the immunogenicity/alloimmunization potential of rhLF.

The proposed GRAS roadmap includes two clinical studies in healthy adults. The first is a 12-week randomized, controlled, double-blind, parallel-arm study, and the second is an 12-week observational study. The first study will comprise a 4-week (Week 4) exposure period followed by a 4-week (Week 8) washout period and an additional 4-week follow up (Week 12) (Supplementary Fig. 5) to evaluate the safety of exposure to two different intakes of Helaina rhLF compared to bLF as the active control. The expert panel agreed that the study duration is sufficient to assess the development of anti-LF antibodies and follows the FDA guidance on assessing immunogenicity/alloimmunization, which recommends testing approximately 30 days of oral exposure (US Food and Drug Administration, 2019). Although this guidance is from the Center for Drug Evaluation and Research (CDER) and the Center for Biologic Evaluation and Research (CBER), Although, this guidance is from CDER/CBER, assessing antibody development against Effera™ as a food ingredient follows the same immunological principles for antibody development following oral delivery of a pharmaceutical. Helaina rhLF will be intended for food applications only, considering it is a dietary protein in the context of human milk consumption. This guidance is referenced because the Center for Food Safety and Applied Nutrition does not have specific guidance on assessing this immunogenicity/alloimmunization safety outcome (Center for Food Safety and Applied Nutrition, 2017). Another instance where food companies used guidance from CBER was noted in both of the submissions for cultured animal cells to the Human Foods Program. Both companies relied on CBER's guidance on procedures for use in culturing (animal/human) cells (US FDA, 2023, 2022). The primary endpoint is serum anti-LF antibodies. Additional endpoints include immune cell proliferation in PBMC with and without re-stimulation with LF, and high-sensitivity C-reactive protein. Safety and tolerability are assessed by evaluating treatment-emergent adverse events and the hematology profile for iron homeostasis (e.g., ferritin, soluble transferrin receptor, transferrin saturation, serum iron) (Supplementary Fig. 6).

Data on anti-LF antibody levels in healthy adults has previously been published (Nässberger et al., 1994; Okazaki et al., 2000; Tan et al., 2014). However, because the variability of anti-LF antibodies over time in the absence of supplemental rhLF among healthy adults is not well characterized, an 12-week observational study is suggested. The timepoints to measure serum anti-LF antibodies are at baseline and weeks 4, 8 and 12 (Supplementary Fig. 7 and Supplementary Fig. 8) to correspond with the rhLF exposure study. Results from this study will help to interpret the findings of the 4-week exposure randomized controlled

Table 2
Key Studies of Helaina rhLF Expressed in *Komagataella phaffii* in the Context of Existing Literature.

Study	Key Safety Area	New to Public Literature (Specific Area)	Corroborates Public Literature (References)	Supports Safety of Effera™ as a Food Ingredient
Allergenicity assessment	Protein Allergenicity *K. Phaffii* Allergenicity Glycan Allergenicity	Yes (Glycan Allergenicity)	*K. phaffii* Allergenicity (Jin et al., 2018) Protein Allergenicity (Zhou et al., 2013)	Yes
Pepsin digestion	Protein Allergenicity	Yes (rhLF from *K. phaffii*)	Protein Allergenicity (Zhou et al., 2013)	Yes
In vitro adult digestion (INFOGEST Protocol)	Digestion	Yes (Digestion)	ADME (Vishwanath-Deutsch et al., 2024)	Yes
28-d rat study	Immunotoxicology General safety and tolerance Iron homeostasis Toxicokinetics	Yes (Immunotoxicology)	ADME, toxicokinetics, general safety and tolerance (Vishwanath-Deutsch et al., 2024)	Yes
Human in vitro/ex vivo cell studies	Immunogenicity risk prediction	Yes (Immunogenicity)	N/A	Yes
Adult clinical study	Alloimmunization/ Immunogenicity Iron homeostasis Safety, Tolerance, and AE	Yes (Alloimmunization)	Safety, Tolerance, and AE (Vishwanath-Deutsch et al., 2024) Iron Homeostasis (Zhao et al., 2022)	Yes

ADME = absorption, distribution, metabolism, excretion; AE = adverse events; N/A = not applicable.

trial.

6. Conclusion

The proposed studies vetted by an expert panel with relevant expertise that will support the GRAS determination of Effera™ are presented in Table 1 and answer the safety questions raised by a 2008 expert panel at the Toxicology Forum. Additionally, the key safety area/s of each pivotal study, the new safety data being added to the public literature as a result of the respective study, and corroborative studies in context of the existing literature are shown in Table 2.With a GRAS study roadmap that directly addresses rhLF-specific safety questions, a comprehensive safety narrative for its use in food is achievable. The goal of the workshop was to convene a panel made up of relevant leading scientists who are experts in their fields of study (Dr. Lukacs – immunology and allergy; Drs. Burns Naas and Kaminski – immunotoxicology; Drs. DiNovi and Merker – food safety assessment; and Dr. Lönnerdal – LF science and nutrition) to build and vet a safety study roadmap that details in a stepwise fashion the types of studies, endpoints, and data needed to answer unanswered questions on immune safety and achieve reasonable certainty of no harm and general recognition among qualified experts (Table 1). While this roadmap is intended to support GRAS for Helaina rhLF (Effera™), when viewed broadly, it can be applied to assess the safety and support GRAS of other recombinant human milk proteins, particularly those that have immunomodulatory functions. Although precision fermentation has been used for decades to make various food components (e.g., vitamins, enzymes) (Hanlon and Sewalt, 2021), it is not until recently that precision fermentation has been used to make bioactive ingredients (e.g., human milk oligosaccharides). It is a burgeoning area of food ingredient innovation that allows bioengineered production organisms to produce high-value ingredients at a commercial scale. Companies may use this roadmap to answer similar questions on the safety of any novel protein food ingredient—derived from precision fermentation or from it native source— with comparable effects on the immune system and intended for adult use. While this roadmap may not answer every potential safety concern of rhLF, nor does it need to per the GRAS safety standard of reasonable certainty of no harm, it does answer the pivotal unanswered safety questions of rhLF required to indicate its safe use as a food ingredient.

Funding

The preparation of this manuscript was supported by Helaina Inc.

CRediT authorship contribution statement

Carrie-Anne Malinczak: Writing – review & editing, Writing – original draft, Conceptualization. **Leigh Ann Burns Naas:** Writing – review & editing, Conceptualization. **Anthony Clark:** Writing – review & editing. **Dietrich Conze:** Writing – review & editing. **Michael DiNovi:** Writing – review & editing. **Norbert Kaminski:** Writing – review & editing. **Claire Kruger:** Writing – review & editing. **Bo Lönnerdal:** Writing – review & editing. **Nicholas W. Lukacs:** Writing – review & editing. **Robert Merker:** Writing – review & editing. **Ross Peterson:** Writing – review & editing, Writing – original draft, Project administration, Conceptualization.

Declaration of competing interest

Carrie-Anne Malinczak, Anthony Clark, and Ross Peterson are employees of Helaina Inc. Leigh Ann Burns Naas, Michael DiNovi, Norbert Kaminski, Bo Lönnerdal, and Robert Merker have received honoraria and/or paid consultancy from Helaina Inc. Dietrich Conze and Claire Kruger are employed by Spherix and have received paid consultancy from Helaina Inc.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors thank Carolyn Alish from Sterling Medical Communications LLC, for her writing and editing assistance on this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114817.

References

Appel, M.J., van Veen, H.A., Vietsch, H., Salaheddine, M., Nuijens, J.H., Ziere, B., de Loos, F., 2006. Sub-chronic (13-week) oral toxicity study in rats with recombinant human lactoferrin produced in the milk of transgenic cows. Food Chem. Toxicol. 44, 964–973. https://doi.org/10.1016/j.fct.2005.11.012.

Bray-French, K., Hartman, K., Steiner, G., Marban-Doran, C., Bessa, J., Campbell, N., Martin-Facklam, M., Stubenrauch, K.-G., Solier, C., Singer, T., Ducret, A., 2021. Managing the impact of immunogenicity in an era of immunotherapy: from bench to bedside. J. Pharmaceut. Sci. 110, 2575–2584. https://doi.org/10.1016/j.xphs.2021.03.027.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.R., Martins, C., Marze, S., McClements, D.J., Ménard, O., Minekus, M., Portmann, R., Santos, C.N., Souchon, I., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Recio, I., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14, 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Center for Food Safety and Applied Nutrition, 2017. Regulatory framework for substances intended for use in human food or animal food on the basis of the generally recognized as safe (GRAS) provision of the federal food, drug, and cosmetic act: guidance for industry. US Food and Drug Administration.

Cerven, D., DeGeorge, G., Bethell, D., 2008a. 28-day repeated dose oral toxicity of recombinant human apo-lactoferrin or recombinant human lysozyme in rats. Regul. Toxicol. Pharmacol. 51, 162–167. https://doi.org/10.1016/j.yrtph.2008.03.007.

Cerven, D., DeGeorge, G., Bethell, D., 2008b. 28-day repeated dose oral toxicity of recombinant human holo-lactoferrin in rats. Regul. Toxicol. Pharmacol. 52, 174–179. https://doi.org/10.1016/j.yrtph.2008.07.003.

Cooper, C., Maga, E., Murray, J., 2014a. Consumption of transgenic milk containing the antimicrobials lactoferrin and lysozyme separately and in conjunction by 6-week-old pigs improves intestinal and systemic health. J. Dairy Res. 81, 30–37. https://doi.org/10.1017/S0022029913000575.

Cooper, C., Nelson, K., Maga, E., Murray, J.D., 2013. Consumption of transgenic cows' milk containing human lactoferrin results in beneficial changes in the gastrointestinal tract and systemic health of young pigs. Transgenic Res. 22, 571–578. https://doi.org/10.1007/s11248-012-9662-7.

Cooper, C., Nonnecke, E., Lönnerdal, B., Murray, J., 2014b. The lactoferrin receptor may mediate the reduction of eosinophils in the duodenum of pigs consuming milk containing recombinant human lactoferrin. Biometals. https://doi.org/10.1007/s10534-014-9778-8.

Digumarti, R., Wang, Y., Raman, G., Doval, D.C., Advani, S.H., Julka, P.K., Parikh, P.M., Patil, S., Nag, S., Madhavan, J., Bapna, A., Ranade, A.A., Varadhachary, A., Malik, R., 2011. A randomized, double-blind, placebo-controlled, phase II study of oral talactoferrin in combination with carboplatin and paclitaxel in previously untreated locally advanced or metastatic non-small cell lung cancer. J. Thorac. Oncol. https://doi.org/10.1097/JTO.0b013e3182156250.

EU Commission, 2012b. Commission Implementing Decision of 22 November 2012 Authorising the Placing on the Market of Bovine Lactoferrin as a Novel Food Ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (FrieslandCampina) (Notified under Document C(2012) 8404).

EU Commission, 2012a. Commission Implementing Decision of 22 November 2012 Authorising the Placing on the Market of Bovine Lactoferrin as a Novel Food Ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (Morinaga) (Notified under Document C(2012) 8390).

Farhangnia, P., Akbarpour, M., 2022. Immunological tolerance. In: Encyclopedia of Infection and Immunity. Elsevier, Oxford, pp. 206–220. https://doi.org/10.1016/B978-0-12-818731-9.00165-8.

Fujiyama, K, Sakai, Y, Misaki, R, Yanagihara, I, Honda, T, Anzai, H, Seki, T, 2004 Dec. N-linked glycan structures of human lactoferrin produced by transgenic rice. Biosci. Biotechnol. Biochem. 68 (12), 2565–2570. https://doi.org/10.1271/bbb.68.2565. PMID: 15618628.

Goldman, A.S., Garza, C., Schanler, R.J., Goldblum, R.M., 1990. Molecular forms of lactoferrin in stool and urine from infants fed human milk. Pediatr. Res. 27, 252–255. https://doi.org/10.1203/00006450-199003000-00009.

Goodman, R.E., Ebisawa, M., Ferreira, F., Sampson, H.A., van Ree, R., Vieths, S., Baumert, J.L., Bohle, B., Lalithambika, S., Wise, J., Taylor, S.L., 2016. AllergenOnline: a peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity. Mol. Nutr. Food Res. 60, 1183–1198. https://doi.org/10.1002/mnfr.201500769.

Guntupalli, K., Dean, N., Morris, P.E., Bandi, V., Margolis, B., Rivers, E., Levy, M., Lodato, R.F., Ismail, P.M., Reese, A., Schaumberg, J.P., Malik, R., Dellinger, R.P., TLF LF-0801 Investigator Group, 2013. A phase 2 randomized, double-blind, placebo-controlled study of the safety and efficacy of talactoferrin in patients with severe sepsis. Crit. Care Med. 41, 706–716. https://doi.org/10.1097/CCM.0b013e3182741551.

Guo, S., Frazer, D.M., Anderson, G.J., 2016. Iron homeostasis: transport, metabolism, and regulation. Curr. Opin. Clin. Nutr. Metab. Care 19, 276–281. https://doi.org/10.1097/MCO.0000000000000285.

Guttner, Y., Windsor, H.M., Viiala, C.H., Marshall, B.J., 2003. Human recombinant lactoferrin is ineffective in the treatment of human Helicobacter pylori infection. Aliment. Pharmacol. Ther. 17, 125–129. https://doi.org/10.1046/j.1365-2036.2003.01395.x.

Hanlon, P., Sewalt, V., 2021. GEMs: genetically engineered microorganisms and the regulatory oversight of their uses in modern food production. Crit. Rev. Food Sci. Nutr. 61, 959–970. https://doi.org/10.1080/10408398.2020.1749026.

Hayes, T.G., Falchook, G.F., Varadhachary, G.R., Smith, D.P., Davis, L.D., Dhingra, H.M., Hayes, B.P., Varadhachary, A., 2006. Phase I trial of oral talactoferrin alfa in refractory solid tumors. Invest. N. Drugs 24, 233–240. https://doi.org/10.1007/s10637-005-3690-6.

Hayes, T.G., Falchook, G.S., Varadhachary, A., 2010. Phase IB trial of oral talactoferrin in the treatment of patients with metastatic solid tumors. Invest. N. Drugs 28, 156–162. https://doi.org/10.1007/s10637-009-9233-9.

Hendrickson, J.E., Tormey, C.A., 2016. Understanding red blood cell alloimmunization triggers. Hematology 446–451. https://doi.org/10.1182/asheducation-2016.1.446, 2016.

Hennart, P.F., Brasseur, D.J., Delogne-Desnoeck, J.B., Dramaix, M.M., Robyn, C.E., 1991. Lysozyme, lactoferrin, and secretory immunoglobulin A content in breast milk: influence of duration of lactation, nutrition status, prolactin status, and parity of mother. Am. J. Clin. Nutr. 53, 32–39. https://doi.org/10.1093/ajcn/53.1.32.

Hinton, D.M., 2000. US FDA "redbook II" immunotoxicity testing guidelines and research in immunotoxicity evaluations of food chemicals and new food proteins. Toxicol. Pathol. 28, 467–478. https://doi.org/10.1177/019262330002800318.

Hu, W., Zhao, J., Wang, Jianwu, Yu, T., Wang, Jing, Li, N., 2012. Transgenic milk containing recombinant human lactoferrin modulates the intestinal flora in piglets. Biochem. Cell. Biol. 90, 485–496. https://doi.org/10.1139/o2012-003.

Hutchens, T.W., Henry, J.F., Yip, T.T., Hachey, D.L., Schanler, R.J., Motil, K.J., Garza, C., 1991. Origin of intact lactoferrin and its DNA-binding fragments found in the urine of human milk-fed preterm infants. Evaluation by stable isotopic enrichment. Pediatr. Res. 29, 243–250. https://doi.org/10.1203/00006450-199103000-00005.

Jawa, V., Cousens, L.P., Awwad, M., Wakshull, E., Kropshofer, H., De Groot, A.S., 2013. T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation. Clin. Immunol. 149, 534–555. https://doi.org/10.1016/j.clim.2013.09.006.

Jin, Y., He, X., Andoh-Kumi, K., Fraser, R.Z., Lu, M., Goodman, R.E., 2018. Evaluating potential risks of food allergy and toxicity of soy leghemoglobin expressed in *Pichia pastoris*. Mol. Nutr. Food Res. 62, 1700297 https://doi.org/10.1002/mnfr.201700297.

Jonasch, E., Stadler, W.M., Bukowski, R.M., Hayes, T.G., Varadhachary, A., Malik, R., Figlin, R.A., Srinivas, S., 2008. Phase 2 trial of talactoferrin in previously treated patients with metastatic renal cell carcinoma. Cancer 113, 72–77. https://doi.org/10.1002/cncr.23519.

Kurtzman, CP., 2005 Mar. Description of Komagataella phaffii sp. nov. and the transfer of Pichia pseudopastoris to the methylotrophic yeast genus Komagataella. Int. J. Syst. Evol. Microbiol. 55 (Pt 2), 973–976. https://doi.org/10.1099/ijs.0.63491-0. PMID: 15774694.

Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T., Hayasawa, H., 2001. Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. J. Nutr. 131, 2121–2127. https://doi.org/10.1093/jn/131.8.2121.

Laffan, A.M., McKenzie, R., Forti, J., Conklin, D., Marcinko, R., Shrestha, R., Bellantoni, M., Greenough, W.B., 2011. Lactoferrin for the prevention of post-antibiotic diarrhoea. J. Health Popul. Nutr. 29, 547–551. https://doi.org/10.3329/jhpn.v29i6.9889.

Laskow, T., Langdon, J., Abadir, P., Xue, Q.-L., Walston, J., 2021. Lactoferrin for the treatment of age-associated inflammation – a pilot study. Phys. Int. https://doi.org/10.1556/2060.2021.00010.

Le Parc, A., Karav, S., Rouquié, C., Maga, E.A., Bunyatratchata, A., Barile, D., 2017. Characterization of recombinant human lactoferrin N-glycans expressed in the milk of transgenic cows. PLoS One 12, e0171477. https://doi.org/10.1371/journal.pone.0171477.

Li, Q., Hu, W., Zhao, J., Wang, J., Dai, Y., Zhao, Y., Meng, Q., Li, N., 2014. Supplementation transgenic cow's milk containing recombinant human lactoferrin enhances systematic and intestinal immune responses in piglets. Mol. Biol. Rep. 41, 2119–2128. https://doi.org/10.1007/s11033-014-3061-5.

Lönnerdal, B., 2013. Bioactive proteins in breast milk. J. Paediatr. Child Health 49 (Suppl. 1), 1–7. https://doi.org/10.1111/jpc.12104.

Lu, X., Cummings, C., Osuala, U.A., Yennawar, N., Namitz, K.E.W., Hellner, B., Besada-Lombana, P.B., Peterson, R.D., Clark, A.J., 2024. Characterization of recombinant human lactoferrin expressed in Komagataella phaffii. Analyst. https://doi.org/10.1039/D4AN00333K.

Manzoni, P., 2016. Clinical benefits of lactoferrin for infants and children. J. Pediatr. 173, S43–S52. https://doi.org/10.1016/j.jpeds.2016.02.075.

Morissette, R., Mihalov, J., Carlson, S.J., Kaneko, K.J., 2023. Trends in ingredients added to infant formula: FDA's experiences in the GRAS notification program. Food Chem. Toxicol. 113876 https://doi.org/10.1016/j.fct.2023.113876.

Nässberger, L., Hultquist, R., Sturfelt, G., 1994. Occurrence of anti-lactoferrin antibodies in patients with systemic lupus erythematosus, hydralazine-induced lupus, and rheumatoid arthritis. Scand. J. Rheumatol. 23, 206–210. https://doi.org/10.3109/03009749409103062.

Nyborg, A.C., Ward, C., Zacco, A., Chacko, B., Grinberg, L., Geoghegan, J.C., Bean, R., Wendeler, M., Bartnik, F., O'Connor, E., Gruia, F., Iyer, V., Feng, H., Roy, V., Berge, M., Miner, J.N., Wilson, D.M., Zhou, D., Nicholson, S., Wilker, C., Wu, C.Y., Wilson, S., Jermutus, L., Wu, H., Owen, D.A., Osbourn, J., Coats, S., Baca, M., 2016. A therapeutic uricase with reduced immunogenicity risk and improved development properties. PLoS One 11, e0167935. https://doi.org/10.1371/journal.pone.0167935.

Office of the Federal Register, National Archives and Records Administration, 2023. Food Additives, Subpart A - General Provisions (No. 21 CFR Sect 170.3), Code of Federal Regulations. Department of Health and Human Services, Silver Spring, MD.

Ofori-Anti, A.O., Ariyarathna, H., Chen, L., Lee, H.L., Pramod, S.N., Goodman, R.E., 2008. Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. Regul. Toxicol. Pharmacol. 52, 94–103. https://doi.org/10.1016/j.yrtph.2008.06.006.

Okazaki, K., Uchida, K., Ohana, M., Nakase, H., Uose, S., Inai, M., Matsushima, Y., Katamura, K., Ohmori, K., Chiba, T., 2000. Autoimmune-related pancreatitis is associated with autoantibodies and a Th1/Th2-type cellular immune response. Gastroenterology 118, 573–581. https://doi.org/10.1016/s0016-5085(00)70264-2.

Opekun, A.R., El-Zaimaity, H.M., Osato, M.S., Gilger, M.A., Malaty, H.M., Terry, M., Headon, D.R., Graham, D.Y., 1999. Novel therapies for Helicobacter pylori infection. Aliment. Pharmacol. Ther. 13, 35–42. https://doi.org/10.1046/j.1365-2036.1999.00435.x.

Parikh, P.M., Vaid, A., Advani, S.H., Digumarti, R., Madhavan, J., Nag, S., Bapna, A., Sekhon, J.S., Patil, S., Ismail, P.M., Wang, Y., Varadhachary, A., Zhu, J., Malik, R., 2011. Randomized, double-blind, placebo-controlled phase II study of single-agent oral talactoferrin in patients with locally advanced or metastatic non-small-cell lung cancer that progressed after chemotherapy. J. Clin. Oncol. 29, 4129–4136. https://doi.org/10.1200/JCO.2010.34.4127.

Perdijk, O., Van Neerven, R.J.J., Van den Brink, E., Savelkoul, H.F.J., Brugman, S., 2018. Bovine lactoferrin modulates dendritic cell differentiation and function. Nutrients 10, 848. https://doi.org/10.3390/nu10070848.

Sherman, M.P., Adamkin, D.H., Niklas, V., Radmacher, P., Sherman, J., Wertheimer, F., Petrak, K., 2016. Randomized controlled trial of talactoferrin oral solution in preterm infants. J. Pediatr. https://doi.org/10.1016/j.jpeds.2016.04.084.

Siegel, M., Steiner, G., Franssen, L.C., Carratu, F., Herron, J., Hartman, K., Looney, C.M., Ducret, A., Bray-French, K., Rohr, O., Hickling, T.P., Smith, N., Marban-Doran, C., 2022. Validation of a dendritic cell and CD4+ T cell restimulation assay contributing to the immunogenicity risk evaluation of biotherapeutics. Pharmaceutics 14, 2672. https://doi.org/10.3390/pharmaceutics14122672.

Sortino, O., Hullsiek, K.H., Richards, E., Rupert, A., Schminke, A., Tetekpor, N., Quinones, M., Prosser, R., Schacker, T., Sereti, I., Baker, J.V., 2019. The effects of recombinant human lactoferrin on immune activation and the intestinal microbiome among persons living with human immunodeficiency virus and receiving antiretroviral therapy. J. Infect. Dis. 219, 1963–1968. https://doi.org/10.1093/infdis/jiz042.

Spadaro, M., Caorsi, C., Ceruti, P., Varadhachary, A., Forni, G., Pericle, F., Giovarelli, M., 2008. Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells. Faseb. J. 22, 2747–2757. https://doi.org/10.1096/fj.07-098038.

Spik, G., Brunet, B., Mazurier-Dehaine, C., Fontaine, G., Montreuil, J., 1982. Characterization and properties of the human and bovine lactotransferrins extracted from the faeces of newborn infants. Acta Paediatr. Scand. 71, 979–985. https://doi.org/10.1111/j.1651-2227.1982.tb09560.x.

Takeuchi, T., Kitagawa, H., Harada, E., 2004. Evidence of lactoferrin transportation into blood circulation from intestine via lymphatic pathway in adult rats. Exp. Physiol. 89, 263–270. https://doi.org/10.1113/expphysiol.2003.026633.

Tan, L., Zhang, Y., Peng, W., Chen, J., Li, H., Ming, F., 2014. Detection of anti-lactoferrin antibodies and anti-myeloperoxidase antibodies in autoimmune hepatitis: a retrospective study. J Immunoassay Immunochem. https://doi.org/10.1080/15321819.2013.879450.

Tao, A., Raz, E., 2015. Allergy Bioinformatics, Translational Bioinformatics. Springer, Dordrdrecht.

Thomas, K., Aalbers, M., Bannon, G.A., Bartels, M., Dearman, R.J., Esdaile, D.J., Fu, T.J., Glatt, C.M., Hadfield, N., Hatzos, C., Hefle, S.L., Heylings, J.R., Goodman, R.E., Henry, B., Herouet, C., Holsapple, M., Ladics, G.S., Landry, T.D., MacIntosh, S.C., Rice, E.A., Privalle, L.S., Steiner, H.Y., Teshima, R., Van Ree, R., Woolhiser, M., Zawodny, J., 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul. Toxicol. Pharmacol. 39, 87–98. https://doi.org/10.1016/j.yrtph.2003.11.003.

Troost, F.J., Saris, W.H.M., Brummer, R.-J.M., 2003. Recombinant human lactoferrin ingestion attenuates indomethacin-induced enteropathy in vivo in healthy volunteers. Eur. J. Clin. Nutr. 57, 1579–1585. https://doi.org/10.1038/sj.ejcn.1601727.

Troost, F.J., Saris, W.H.M., Brummer, R.-J.M., 2002. Orally ingested human lactoferrin is digested and secreted in the upper gastrointestinal tract in vivo in women with ileostomies. J. Nutr. 132, 2597–2600. https://doi.org/10.1093/jn/132.9.2597.

Troost, F.J., Steijns, J., Saris, W.H., Brummer, R.J., 2001. Gastric digestion of bovine lactoferrin in vivo in adults. J. Nutr. 131, 2101–2104. https://doi.org/10.1093/jn/131.8.2101.

US FDA, 2023a. GRN No. 1056. β-lactoglobulin Produced by Komagataella Phaffii Strain "yRMK-66.".

US FDA, 2023b. GRN No. 1025. Pepsin A Enzyme Preparation Produced by Pichia pastoris to Overexpress the Gene Encoding Pepsin A.

US FDA, 2021a. GRN No. 967. Soluble Egg-White Protein Produced by Komagataella Phaffii Strain GSD-1209.

US FDA, 2021b. GRN No. 1001. Myoglobin Preparation from a Strain of Pichia pastoris Expressing the Myoglobin Gene from Bos taurus.

US FDA, 2018. GRN No. 737. Soy Leghemoglobin Preparation from a Strain of Pichia pastoris.

US FDA, 2017. GRN No. 669. Cow's Milk-Derived Lactoferrin.

US FDA, 2014a. GRN No. 464. Cow's Milk-Derived Lactoferrin.

US FDA, 2014b. GRN No. 465. Cow's Milk-Derived Lactoferrin.

US FDA, 2007. GRN No. 235. Human Lactoferrin Purified from Rice.

US FDA, 2006. GRN No. 204. Phospholipase C Enzyme Preparation from Pichia pastoris Expressing a Heterologous Phospholipase C Gene.

US FDA, 2005. GRN No. 189. Lactoferrin (Human) Purified from Bovine Milk.

US FDA, 2004. GRN No. 162. Lactoferrin (Human) Purified from Rice.

US FDA, 2001. GRN No. 77. Milk-Derived Lactoferrin.

US Food and Drug Administration, 2022. Best practices for convening a GRAS panel, pp. 8–12. Guidance Indus.

US Food and Drug Administration, 2021. Non-clinical immunogenicity assessment of generic peptide products: development, validation, and sampling - 01/26/2021 - 01/26/2021 [WWW Document]. FDA URL. https://www.fda.gov/drugs/news-events-human-drugs/non-clinical-immunogenicity-assessment-generic-peptide-products-development-validation-and-sampling, 9.12.22.

US Food and Drug Administration, 2019. Immunogenicity testing of therapeutic protein products —developing and validating assays for anti-drug antibody. Detection Guidance Indus.

US Food and Drug Administration, 2007. Guidance for Industry and Other Stakeholders: Redbook 2000. Toxicological Principles for the Safety Assessment of Food Ingredients, pp. 234–252.

Vincent, J.-L., Marshall, J.C., Dellinger, R.P., Simonson, S.G., Guntupalli, K., Levy, M.M., Singer, M., Malik, R., 2015. Talactoferrin in severe sepsis: results from the phase II/III Oral tAlactoferrin in Severe sepsIS Trial. Crit. Care Med. 43, 1832–1838. https://doi.org/10.1097/CCM.0000000000001090.

Vishwanath-Deutsch, R., Dallas, D.C., Besada-Lombana, P., Katz, L., Conze, D., Kruger, C., Clark, A.J., Peterson, R., Malinczak, C.-A., 2024. A review of the safety evidence on recombinant human lactoferrin for use as a food ingredient. Food Chem. Toxicol. 189, 114727 https://doi.org/10.1016/j.fct.2024.114727.

Zavaleta, N., Figueroa, D., Rivera, J., Sánchez, J., Alfaro, S., Lönnerdal, B., 2007. Efficacy of rice-based oral rehydration solution containing recombinant human lactoferrin and lysozyme in Peruvian children with acute diarrhea. J. Pediatr. Gastroenterol. Nutr. 44, 258–264. https://doi.org/10.1097/MPG.0b013e31802c41b7.

Zhao, X., Zhang, X., Xu, T., Luo, J., Luo, Y., An, P., 2022. Comparative effects between oral lactoferrin and ferrous sulfate supplementation on iron-deficiency anemia: a comprehensive review and meta-analysis of clinical trials. Nutrients 14, 543. https://doi.org/10.3390/nu14030543.

Zhou, C., Sun, N., Wang, J., Lu, J., Tian, J., Goodman, R.E., Li, N., Che, H., Huang, K., 2013. Allergenicity assessment of a genetically modified protein-recombinant human lactoferrin. J. Allergy Ther. 0, 1–6. https://doi.org/10.4172/2155-6121.S3-002.

Zhou, C., Wang, J.W., Huang, K.L., He, X., Chen, X.P., Sun, H., Yu, T., Che, H.L., 2011. A 90-day safety study in Sprague-Dawley rats fed milk powder containing recombinant human lactoferrin (rhLF) derived from transgenic cloned cattle. Drug Chem. Toxicol. 34, 359–368. https://doi.org/10.3109/01480545.2010.542465.